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QUANTITATIVE STUDIES OF CELL TYPES IN THE RAT HYPOPHYSIS FOLLOWING UNILATERAL ADRENALECTOMY

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THE INTERRELATIONSHIP between hypophysis and adrenal cortex has been established conclusively by extensive experimentation with hypophysectomized animals, through the use of pituitary implants and by means of injections of extracts into both intact and hypophysectomized animals. Reduction of adrenal cortical hormone concentration in the blood is believed to increase hypophyseal adrenotrophic secretion, resulting in hypertrophy and hypersecretion of the adrenal cortex (Tepperman, Engel and Long, 1943). Nevertheless, in spite of remarkable agreement among present investigators concerning the relationship between pituitary gland and adrenal cortex, there is lack of agreement regarding the specific type of cell in the anterior hypophysis which is responsible for secretion of the adrenocorticotrophic hormone. Until recently, most studies of this problem had been limited to observations on the histology of the pituitary gland following death from Addison's disease (Kraus, 1927), or in the terminal stages of complete adrenalectomy of experimental animals. The most common findings under these conditions have been diminution and degeneration of both acidophiles and basophiles. Crooke and Russell (1935) state that the constant features in pathological adrenal insufficiency are the extreme decline in the number of basophile cells, a variable reduction in the number of acidophiles, the presence of a series of abnormal basophilic cells and of a considerable number of large chromophobes. Severinghaus (1938) describes one case of a woman with Addison's disease in which there was a marked increase in basophiles. In summarizing the results in this field, Severinghaus states, "Addison's disease in general seems to be associated with a depletion of basophiles from the hypophysis, but certain glands have

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appeared to show an attempt to attain normalcy, a process which may be of such short duration as to be generally missed. Basophilic activity seems indicated." Reese, Koneff and Akimoto (1939), in studying the anterior pituitary glands of male rats showing acute symptoms after double adrenal ablation, find diminution in number and size of acidophiles, correlated with progressive loss of granular material and regressive changes in the Golgi apparatus. The basophiles of their rats are also obviously diminished in number and size with cytological changes being dependent upon severity of symptoms and length of post-operative period. The observations, upon which these conclusions are based, were on terminal stages of bilateral adrenalectomy in experimental animals, or on cases of Addison's disease; thus the question arises as to whether the changes were a result of modification in pituitary-adrenal cortical relationship, or of abnormal metabolism of the body as a whole (inanition?).

Heinbecker and Rolf (1944) attribute adrenotrophic secretion to the eosinophilic cells: they find functional depression of the adrenal cortex followed by progressive atrophy in hypophysectomized dogs, whereas when the infundibular stem is severed and fibers from the paraventricular nucleus are cut by a puncture wound in the posterior hypothalamus the eosinophile cells are maintained, the basophiles decrease in number and the adrenal cortex remains normal.

D'Angelo, Gordon and Charipper, (1948) have utilized the characteristic absolute adrenal hypertrophy of guinea pigs following deprivation of food as a means of determining the cellular basis for adrenocorticotrophic secretion. Under these conditions of probable corticotrophic hypersecretion they find progressive loss of acidophilia, and an increase in number of basophiles and chromophobes. "These results are interpreted to mean that cortical hypertrophy in the starving guinea pig results from augmentation of adrenotrophin secretion by the basophiles of the anterior pituitary." Unfortunately, the cortical hypertrophy of inanition and the cellular changes in the hypophysis have not been produced in animals other than guinea pigs, and the cortical hypertrophy seems to be unaffected by exogenous cortical hormones.

The present observations are on unilaterally adrenalectomized rats, in which relative reduction in adrenal cortical tissue stimulated adrenocorticotrophic activity so that compensatory hypertrophy of the remaining adrenal gland occurred. These animals were healthy, well-nourished and continued to grow so that their body weights were only slightly lower than those of unoperated control rats.

PROCEDURE

The results of this investigation are based on quantitative histological studies of the pituitary and adrenal glands of one hundred and twenty male rats of the Sprague-Dawley strain. Male rats were used to avoid the compli-

cations of any cyclic phenomena associated with the estrous cycle. The left adrenal glands were removed from all experimental animals at the age of 31 days. One group was kept at laboratory temperature (Approx. 26°C.), and a second group was placed in a cold room kept at 5.6°C. to apply an additional stimulus to compensatory hypertrophy. Autopsies were performed 5, 10, 15, and 25 days following operation. The hypophysis was removed and immediately fixed in Zenker-formol solution. Adrenal glands, thyroid gland, thymus and testes were weighed on a torsion balance and fixed in 10% formalin.

The pituitary glands were sectioned in a horizontal plane at 4 micra and stained with a modified azocarmine stain (Briseno-Castrejon and Finerty, 1949). In this method the nuclei were pre-stained by a short immersion in alum hematoxylin. This was followed by mordanting in aniline alcohol and a 45 minute period in azocarmine solution at 60°C. The counterstains, acid green and orange G, were dissolved in clove oil to avoid destaining of the azocarmine. After the above staining, acidophilic granules are purplish red; basophile granules are light green; nuclear membranes are sharply defined; mitochondria are orange-red; red blood cells are brilliant orange; Golgi apparatus shows as a negative image in both types of chromophiles; and chromophobes show little or no cytoplasm, which is colorless to pale green.

Quantitative determinations of the percentages of each cell type were made by differentially counting all the cells in every fifth field of three horizontal sections at equidistant levels in each gland. Approximately 3000 cells were counted in each gland.

RESULTS AND DISCUSSION

The consistent responses to unilateral adrenalectomy are compensatory hypertrophy of the remaining adrenal gland and increased percentage of acidophiles in the anterior hypophysis, as is shown in the summarized results in Table 1.

In all cases of unilateral adrenalectomy, compensatory hypertrophy of the remaining adrenal gland occurred. Using the mean weight of the right adrenal glands of the unoperated control rats as a base line, the percentage of absolute hypertrophy was calculated. There seems to be no consistent pattern of progressive hypertrophy, except that those rats exposed to the additional stress of a continual cold environment exhibit considerably more adrenal growth. When the basis of proportion of adrenal weight to body weight is considered, the hypertrophy is of a greater degree, especially in the cold-exposed rats, in which there is a greater absolute hypertrophy of the adrenal gland, which is accompanied by a lower body weight than in those kept at room temperature.

Since the adrenal glands appear to respond to the administration of various forms of stress only through the mediation of pituitary adrenotrophic activity (Sayers, Sayers and Woodbury, 1948) it may be assumed that the compensatory hypertrophy is a result of increased pituitary secretion. This assumption is substantiated by the changes which are shown in the percentages of cell types in the anterior hypophysis. In those rats which have been subjected to relative reduction

of circulating adrenal cortical hormones and are undergoing heightened adrenal stimulation, there is a significant increase in the percentage of acidophilic cells. Superficial examination of the hypophysis in unilaterally adrenalectomized rats in some cases conveys the impression that the number of basophiles has increased. However, detailed quantitative analysis demonstrates that there is an unequivocal augmentation in number of acidophiles balanced by a corresponding decrease in chromophobes. The apparent increase in percentage of

TABLE 1. THE EFFECT OF UNILATERAL ADRENALECTOMY AND EXPOSURE TO LOW TEMPERATURE ON THE PERCENTAGE OF CELL TYPES IN THE PITUITARY GLANDS OF IMMATURE MALE RATS

	Days After Operation	No. of Rats	Rt. Adrenal Wt. (mg.)	Adrenal Hyper-Trophy[1] %	Pituitary Cell Type Analysis % Acid	% Baso	% Chrom.
Control		5	12.1		41.7	7.6	50.3
Operated (26°C.)	5	5	15.5	28	47.4	7.3	45.3
Operated (5.6°C.)		5	19.6	62	47.0	6.8	46.2
Control		5	15.0		39.7	8.0	52.3
Operated (26°C.)	10	5	20.0	33	49.9	8.4	41.7
Operated (5.6°C.)		5	21.1	41	51.8	6.7	41.5
Control		25	16.4		37.4	7.5	55.3
Operated (26°C.)	15	25	19.8	21	49.2	7.5	43.3
Operated (5.6°C.)		25	21.7	32	49.6	6.8	43.6
Control		5	16.1		37.9	7.4	54.7
Operated (26°C.)	25	5	19.7	22	53.7	7.8	38.5
Operated (5.6°C.)		5	26.2	63	51.6	8.1	40.3

[1] The percentage of hypertrophy is calculated by using the mean weight of the right adrenal glands of the control rats as the basis for comparison.

basophiles in certain instances is due to their greater variability and to possible decrease in size of individual acidophiles. Statistical analysis of the percentages of acidophiles in the twenty-five control rats of the group studied fifteen days following operation shows a mean percentage of $37.4 \pm .52$.[2] Similar analysis of the twenty-five unilaterally adrenalectomized rats kept at room temperature shows a mean of 49.2 per cent $\pm .78$. The twenty-five unilaterally adrenalectomized rats kept at 5.6° C. show a mean of 49.6 per cent $\pm .61$. It appears that at 5 days following removal of one adrenal gland the changes in pituitary cell types have not reached a maximum, but that there is little progressive change after the 10 day period. The higher percentage in the 25 day group is difficult to interpret but is consistent in that none of the rats in this series had an acidophile count below 50 per cent. Experiments are now in progress to determine if, and when, the pituitary cytologic pattern returns to a normal level following the restoration of adrenal cortical function by the hypertrophied gland.

[2] Standard error.

A graphic presentation of the variability of acidophile cell percentages to demonstrate better the consistency of the data is shown in Figure 1. The 15 day series is shown in which there were 25 rats in each of the three groups. The rats are classified into four-percentile groups: all those with acidophile percentages between 32–35.9; 36–39.9; 40–43.9; etc. are placed in separate columns. It can be seen that the distribution of the control rat percentages (black columns) is

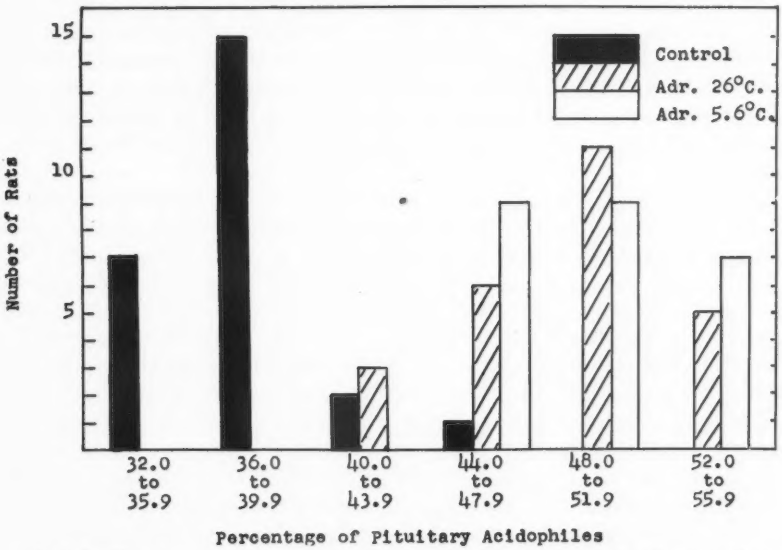

FIG. 1. Histogram to demonstrate graphically the frequency distribution of the percentages of acidophilic cells in the pituitary glands of untreated 46 day old male rats (black columns); 46 day old male rats which had been unilaterally adrenalectomized 15 days previously and kept at laboratory temperature (hatched columns); and 46 day old male rats which had been unilaterally adrenalectomized 15 days previously and kept in a cold room at 5.6°C. (white columns). The abscissa is subdivided into six successive groups of 4 per cent increments, into which are projected the number of rats having an acidophile count within each group. Note that the control rats show the greatest frequency between 36.0 and 39.9 per cent, whereas the adrenalectomized groups cluster about the 48.0 to 51.9 percentage group.

concentrated around the 36–39.9 group; the percentages with greatest frequency in the experimental groups are in the 48–51.9 group.

The basophile cells are the least numerous, ranging from 5.7 to 9.3 per cent in the control animals; from 5.1 to 9.2 per cent in the experimental rats at room temperature; and from 5.3 to 9.7 per cent in the experimental rats exposed to cold. The mean percentages in each group, however, show no effect of the adrenalectomy on the basophiles (Table 1).

No weight or volumetric estimations of the hypophysis were made at autopsy so as to avoid post mortem changes before fixation.

Micrometric measurements of every tenth serial section, however, demonstrate that the hypophyses of the rats exposed to cold were larger than those of animals (control, or adrenalectomized) kept at room temperature. This change was in the anterior lobe, since the pars intermedia and pars nervosa maintained a uniform size. This is in accordance with the observations made by Baillif (1938). Histological observations of the hypophyses of rats exposed to cold also confirm his findings that enlargement of the anterior lobe is due to increase in size of blood sinusoids, more numerous colloid vesicles and possible increase in size of basophiles. Extreme disintegration of the anterior lobe observed by Baillif (1938) under severe exposure to cold was not noted under the present experimental conditions, nor was cellular disintegration evident. Although no specific counts of mitotic index were made, more mitoses seemed to be present in the rats exposed to cold.

The weight of the thymus and of the testes was slightly reduced in the rats of the experimental groups, but when the weights of these organs is considered in relation to body weight it appears to be dependent upon the weight of the animal rather than upon the experimental procedures. The weight of the thyroid gland was unchanged which may indicate a slight hypertrophy in the experimental groups.

Much of the current knowledge concerning histological changes in the anterior lobe of the hypophysis produced by alteration in relationship between hypophysis and adrenal cortex is based on studies of the pituitary gland in Addison's disease, in bilaterally adrenalectomized animals, and to some extent in acromegaly. The sustentative data which are possible under controlled conditions of unilateral adrenalectomy have been ignored. In the histopathology of Addison's disease there is almost unanimous agreement in regarding the basophiles as the characteristic cells involved, even though the same authors describe similar alterations in the acidophiles; viz., a decrease in number of cells, indistinct and irregular cell borders, sparse cytoplasmic granulation and pyknotic nuclei. Histological evidence derived from the study of the anterior lobe after complete ablation of the adrenals has also produced contradictory and confusing results. Shumaker and Firor (1934) in describing the pars distalis of a bilaterally adrenalectomized dog, 128 days after operation, concluded that basophilic alterations, similar to those found in Addison's disease, are characteristic after complete removal of suprarenal glands. The results of Reese, Koneff and Akimoto (1939) also agree with this conclusion. Acromegaly is associated with hyperfunction of the pituitary gland and is characterized by acidophilic hyperplasia, or adenoma, accompanied by hyperplasia of the adrenal cortex. If the acidophilic hyperactivity brings about hyperplasia of the suprarenal tissue, this condition may be indicative that the adrenocorticotrophic principle is secreted by the acidophiles.

Thus, we have two opposite interpretations of the source of adrenocorticotrophin, obtained by different methods. From clinical observations in Addison's disease, the basophiles seem to be most involved (although they are described as showing degenerative effects); in acromegaly there is adrenal enlargement associated with acidophile hyperplasia. Experimentally bilateral adrenalectomy evokes degenerative changes in acidophiles and basophiles, whereas unilateral adrenalectomy produces acidophile predominance. The findings in acromegaly, as in Addison's disease or in bilateral adrenalectomy, may be the result of severe metabolic imbalance. Another possibility to explain these divergent findings, however, is that the presence of some normally functioning adrenal tissue is required for proper functioning of pituitary-adrenal interaction.

Since increased adrenotrophic activity, as indicated by adrenal hypertrophy, results from diminished adrenal cortical tissue, it has been suggested that reduction in circulating adrenal cortical hormone is the stimulus for pituitary adrenotrophic activity. Sayers and Sayers (1947) subjected adult male rats to various forms of stress for one hour and found that each type of stress produced an increase in adrenocorticotrophic activity, as determined by reduction in ascorbic acid content of the adrenal glands. Injection of appropriate amounts of cortical hormone prevented this hypersecretion. They conclude that, "the anterior pituitary elaborates adrenocorticotrophic hormone at a rate inversely proportional to the concentration of cortical hormone(s) in the body fluids and according to the requirements of the peripheral tissue cells for cortical steroids."

The results of the present experiments are interpreted to indicate that the increased adrenotrophic activity which follows reduction in circulating adrenal cortical hormone(s) is mediated by an increase in the number and activity of the acidophilic cells of the hypophysis, and that the source of adrenocorticotrophin is the acidophiles.

SUMMARY

Unilateral adrenalectomy of immature male rats results in compensatory hypertrophy of the remaining adrenal gland and a marked increase in percentage of acidophilic cells in the anterior hypophysis. When unilaterally adrenalectomized rats are subjected to lowered environmental temperatures these changes are accentuated. It is believed that reduction in circulating adrenal hormone, either by removal of adrenal tissue, or by stress, stimulates an increase in number of pituitary acidophiles, and that these cells secrete adrenocorticotrophic hormone.

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THE EFFECT OF HYPNOTICS ON BLOOD SUGAR AND ON THE ACTION OF INSULIN[1]

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THE EFFECT of barbiturates or morphine upon the blood sugar level has been the subject of several investigations but an examination of the results reported does not lead to convincing conclusions as will be evident from the following brief résumé.

Barbital sodium, as reported by Ellis and Barlow (1924), lowered the blood sugar in both cats and pigeons. Weiss (1926) has reported that barbital caused a marked initial rise in blood sugar in the cat and dog which is followed by a gradual fall. Jackson (1931) reported that in rabbits, barbital sodium retards the hypoglycemia produced by insulin. Murphy and Young (1932) have reported that pentobarbital and other barbiturates produced an increase in blood sugar in cats. Jarman and Abel (1933) reported that evipal sodium in humans produced no alteration in blood sugar. Hemingway et al. (1934) found that pentobarbital in the dog caused a slight increase in blood sugar. Goodman and Gilman (1941a) state "The response of the blood sugar to medication with the barbiturates varies considerably." La Barre and Vesselovsky (1942) reported that barbital sodium in dogs caused a fleeting rise in blood sugar.

Morphine, according to Ross (1918), caused a marked hyperglycemia in the dog. Stewart and Rogoff (1922a) report that morphine causes a marked hyperglycemia in the cat with lesser effects in the rabbit and dog. Bodo et al. (1938) reported that in adrenal-inactivated dogs and cats, morphine liberates sympathin which causes a slight hyperglycemia. Goodman and Gilman (1941b) state "The hyperglycemia noted after morphine in animals is inconstant." Meperidine hydrochloride causes a slight increase in the blood sugar of rabbits (Barlow, 1947).

Due to the lack of harmony of these reports, further investigation of the effect of these drugs on the blood sugar level is suggested. Since few of the previous investigators have been concerned with any effect of these drugs on the hypoglycemic action of insulin, this phase will

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[1] Preliminary reports have been presented to the American Society of Pharmacology and Experimental Therapeutics. Federation Proc., 4: 125, 1945 & Ibid. 5: 189, 1946.

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be included in the present study. Since albino rats have a relatively constant blood sugar (Cole and Harned, 1938), they were chosen as the experimental animals.

METHODS

Albino male rats, of the Wistar strain varying in weight from 150 to 350 grams, free from gross evidence of disease, were maintained on a commercial food (considered complete) supplemented by daily feeding with milk, whole wheat bread and lettuce. These animals also were given a few drops of fish oil[3] about every ten days. All food was removed from the cages 15–17 hours before each test began. In order to facilitate the securing of blood samples, the animals were placed in individual cylindrical wire cages with the tails protruding. The animals during the experimental procedures were kept at the temperature designated in Tables 1, 2, 3 & 4. Blood samples were obtained by amputating a thin segment of the tail with sharp scissors and then gently stroking the tail between the fingers, the blood being drawn into a glass capsule containing potassium oxalate. The blood sugar values were determined, after deproteinization (Somogyi, 1930), by either the method of Shaffer and Somogyi (1933) or Somogyi (1937). After taking the first sample which is designated "control," the drugs were injected and four more samples were obtained at successive one-hour intervals. The insulin injections were made subcutaneously and the other drugs were injected intraperitoneally. All injections were made aseptically. The doses mentioned are either units (U.) or milligrams (mg.) of the drugs per kilogram (kg.) of body weight. No animal was used without an interval of at least ten days elapsing between individual experiments.

RESULTS

From the average control blood sugars presented in Tables 1, 3 and 4, it is evident that these values are relatively constant. There is also no significant change in the average blood sugar level in the normal groups over a period of five hours.

Morphine sulfate, at a low dose level (12 mg. Table 1, Group B) had no effect on the blood sugar level except a questionable slight increase at the end of hour 2. With the 25 mg. dose (Table 1, Group C) the average blood sugar level at the end of one hour is normal, but there were marked variations in the individual values. Since hyperglycemia[4] occurred in only 1 case in the 2, 3 or 4 hour blood sample with several cases of hypoglycemia,[5] the averages are below normal. In the 50 mg. group (Table 1, Group D) the highest recorded blood sugar average (82.2) was reached at the end of one hour as a result of 4 animals showing a hyperglycemia despite the fact that 5 animals

[3] Vitamin A and D content equal to Cod Liver Oil U.S.P. XIII.

[4] Individual blood sugar values above 95 mg. per 100 cc. are considered hyperglycemic because the methods used show only "true sugar" and also the animals have been fasted for 15–17 hours which produced normal values of an average range from 63.7–71.5 mg. per 100 cc.

[5] Individual blood sugar values below 45 mg. per 100 cc. are considered hypoglycemic.

TABLE 1. THE EFFECTS OF INSULIN ON BLOOD SUGAR AS INFLUENCED BY HYPNOTICS

The average blood sugar values are expressed as mg. per 100 cc. The number of hyperglycemias are designated + and the hypoglycemias −. The animals were kept in a box maintained at 29°C. ±1° during the experimental procedure. The drugs were injected just after the control sample was obtained. The number of animals that died from this dose before the one hour sample was obtained is designated thus *. These animals were replaced to complete a group of ten in each case.

Group	Experimental Condition	Control	Hour				Addenda
			1	2	3	4	
A	Normal Group	67.8	65.8	67.6	68.2	62.4	
B	Morphine Sulfate 12 mg.	70.5	73.4	81.5	74.8	69.1	
C	Morphine Sulfate 25 mg.	64.6	65.0	59.9	60.4	57.9	*2, +4, −11, 1 death each during hr. 1, 2 & 3
D	Morphine Sulfate 50 mg.	67.8	82.2	60.5	69.0	68.1	*1, +7, −9, 1 death during hr. 1 & 3 during hr. 2
E	Barbital Sodium 100 mg.	67.9	64.3	57.9	60.8	60.4	−1
F	Barbital Sodium 200 mg.	71.2	76.1	66.3	65.3	67.4	−1, 1 death during hr. 3
G	Insulin 1 U.	66.3	21.3	18.7	24.6	36.1	−32, 2 deaths during hr. 3 & 1 during hr. 4
H	Morphine Sulfate 12 mg. & Insulin 1 U.	64.9	20.4	14.8	25.3	38.2	−37
I	Morphine Sulfate 25 mg. & Insulin 1 U.	67.5	22.2	6.1	12.3	17.9	*1, −26, 3 deaths, 1 during hr. 1 & 2 during hr. 2
J	Morphine Sulfate 50 mg. & Insulin 1 U.	64.6	27.9	14.2	8.1	17.6	*1, −26, 6 deaths, 1 during hr. 2 & 5 during hr. 3
K	Barbital Sodium 100 mg. & Insulin 1 U.	68.6	22.8	22.9	23.2	24.4	−38
L	Barbital Sodium 200 mg. & Insulin 1 U.	68.4	32.0	26.3	32.4	38.9	−28. 3 deaths, 2 during hr. 2 & 1 during hr. 3

had a hypoglycemia. There were seven deaths in Groups C and D, apparently from respiratory paralysis.[6] Barbital sodium, 100 or 200 mg. slightly depressed the average blood sugar levels except in one case (Table 1, Groups E and F). One unit of insulin, as would be expected, lowered the blood sugar to hypoglycemic levels and even

[6] The blood sugars of these dead animals, in nearly every instance, were decidedly hypoglycemic but they were not included in the average for that hour because the samples had to be obtained from the heart whereas the other samples were obtained from the tail.

caused the death of three animals (Table 1, Group G). The effect of 12 mg. of morphine sulfate (Table 1, Group H) on the hypoglycemic effect of one unit of insulin did not seem significant but there were no deaths in this group. In larger doses (25 and 50 mg.) morphine sulfate (Table 1, Groups I and J) accentuated the hypoglycemic action of insulin except in two instances (both Hour 1). Three of the 10 animals which received insulin and 25 mg. of morphine sulfate died and also 6 of the 10 receiving insulin and 50 mg. of morphine sulfate died. The hypoglycemic action of insulin was not altered significantly by 100 mg. of barbital sodium (Table 1, Group K) but the 200 mg. dose (Table 1, Group L) did have an antagonistic action. Though the hypoglycemic action was not opposed by the 100 mg. dose there were no deaths in this group as compared with three in each of the other two groups (Table 1, Groups G and L).

TABLE 2. EFFECT OF MORPHINE SULFATE ON RECTAL TEMPERATURE AT DIFFERENT ENVIRONMENTAL TEMPERATURES

Average temperatures of four groups of ten rats each, previously fasted for 15–17 hours. Groups A and B kept at room temperature, 20.2°C., throughout and Groups C and D kept in cabinet at 20.3°C. for hours 0 & 1 and thereafter at 29°C. ±1°.

Group	Hour							Addenda
	0	1	2	3	4	5	6	
A	37.1	36.8	No Injection	36.6	36.4	36.4	36.3	
B	37.7	37.6	Morphine Sulfate 25 mg.	35.2	34.6	37.0	37.1	No deaths
C	37.3	37.3	No Injection	37.4	37.4	37.6	37.6	
D	37.6	37.4	Morphine Sulfate 25 mg.	36.8	36.3	37.0	38.0	3 deaths

Because of the deaths reported in Table 1 when the animals were maintained at 29° C., it seemed advisable to repeat the experiments with morphine at the 25 mg. dose level with a part of the animals maintained at room temperature (20.2° C.) and others at 29° C. When given 25 mg. of morphine sulfate, the group (B) kept at room temperature showed a definite decrease in body temperature whereas the group (D) kept at 29° C. showed only a slight change. It should be noted, however, that in Group D, 3 of the 10 rats died during the four hours following the injection of morphine.

Since the increased environmental temperature augmented the toxicity of morphine, the succeeding experiments summarized in Tables 3 and 4, were performed with only the tail being warmed to facilitate securing of blood samples. The blood sugar values of the corresponding normal Groups A of Tables 1 and 3 are as close as could be expected from two different groups of rats.

The average blood sugar level is not changed significantly (Table 3) by pentobarbital sodium (Groups B and C), evipal sodium (Group

TABLE 3. THE EFFECT OF HYPNOTICS ON BLOOD SUGAR AND RECTAL TEMPERATURE

The average blood sugar results are expressed as mg. per 100 cc. and the average temperature in °C. The number of hyperglycemias observed are designated + and the hypoglycemias −. The bodies of the rats were kept at room temperature but the tails were inserted into a warmed box maintained at 40°C. ±1°. The drugs were injected immediately after the control blood samples were obtained. The number of animals that died before the one hour blood sample was obtained is designated thus *. These animals were replaced to complete a group of ten in each case.

Group	Experimental Condition	Control		Hour								Addenda
				1		2		3		4		
		Blood Sugar	Temp. °C.	Blood Sugar	Temp. °C.	Blood Sugar	Temp. °C.	Blood Sugar	Temp. °C.	Blood Sugar	Temp. °C.	
A	Normal Group	69.7	37.2	66.6	37.3	66.7	37.2	64.9	37.2	65.4	37.5	
B	Pentobarbital Sodium 25 mg.	63.7	37.6	68.4	35.0	62.7	36.0	62.5	36.8	62.4	37.4	*1
C	Pentobarbital Sodium 35 mg.	69.2	37.2	71.7	34.4	64.5	34.9	65.8	36.2	69.3	36.8	*2, 1 death during hr. 2
D	Evipal Sodium 100 mg.	68.8	37.2	71.4	35.2	66.9	36.0	63.9	36.8	64.0	37.4	*5, −1
E	Barbital Sodium 100 mg.	69.9	37.1	66.4	34.6	63.7	34.0	67.2	34.6	66.7	35.2	
F	Barbital Sodium 200 mg.	68.1	37.4	70.4	35.1	65.7	34.1	66.7	33.8	72.5	33.4	4 deaths, 1 during hr. 1, 2 during hr. 2 & 1 during hr. 4
G	Meperidine Hydrochloride 15 mg.	64.7	36.7	62.2	35.4	61.0	36.2	63.9	36.6	66.9	36.6	
H	Meperidine Hydrochloride 30 mg.	71.3	37.4	69.8	35.4	62.2	35.6	65.7	36.5	61.0	37.0	
I	Meperidine Hydrochloride 50 mg.	69.1	37.0	67.2	33.0	72.2	33.6	74.2	34.5	75.5	35.2	*2, −1, +1
J	Morphine Sulfate 12 mg.	68.0	37.2	69.3	36.2	70.0	37.2	69.5	37.9	68.1	37.7	
K	Morphine Sulfate 25 mg.	65.5	37.2	63.8	33.7	72.8	33.5	66.6	35.9	63.2	36.9	
L	Morphine Sulfate 50 mg.	71.2	37.8	64.0	32.9	75.7	32.7	69.2	33.6	68.2	34.7	−3, +4

TABLE 4. THE EFFECT OF INSULIN ON BLOOD SUGAR AND RECTAL TEMPERATURE AS INFLUENCED BY HYPNOTICS

The procedures and designations are the same as those of Table 3.

Group	Experimental Condition	Control		Hour								Addenda
				1		2		3		4		
		Blood Sugar	Temp. °C.	Blood Sugar	Temp. °C.	Blood Sugar	Temp. °C.	Blood Sugar	Temp. °C.	Blood Sugar	Temp. °C.	
A	Insulin 1 U.	68.0	37.6	30.4	36.6	26.4	36.2	32.2	36.1	36.0	36.7	−36
B	Pentobarbital Sodium 25 mg. & Insulin 1 U.	70.3	37.7	33.9	34.4	32.6	34.4	33.5	34.8	37.7	35.4	−36
C	Pentobarbital Sodium 35 mg. & Insulin 1 U.	70.0	37.1	27.9	33.2	33.4	34.0	37.7	34.6	45.4	35.6	*2, −34
D	Evipal Sodium 100 mg. & Insulin 1 U.	69.5	37.4	30.4	33.8	37.1	34.0	34.7	34.5	43.9	34.9	*3, −30, 1 death during hr. 2
E	Barbital Sodium 100 mg. & Insulin 1 U.	69.9	37.1	33.0	33.4	27.4	32.4	36.3	32.3	45.6	32.8	−35
F	Barbital Sodium 200 mg. & Insulin 1 U.	69.6	37.2	30.2	35.3	26.3	34.5	33.2	34.1	41.2	34.5	−31, 4 deaths, 1 during hr. 1, 2 during hr. 2 & 1 during hr. 3
G	Meperidine Hydrochloride 15 mg. & Insulin 1 U.	64.2	36.9	31.4	34.1	29.1	33.7	29.0	33.6	38.3	34.6	−36
H	Meperidine Hydrochloride 30 mg. & Insulin 1 U.	71.3	37.4	20.6	32.4	21.8	31.8	23.6	31.9	23.8	32.6	−38, 2 deaths during hr. 3
I	Meperidine Hydrochloride 50 mg. & Insulin 1 U.	71.3	36.6	34.3	32.3	38.3	31.6	42.3	32.4	43.5	33.8	*1, −31
J	Morphine Sulfate 12 mg. & Insulin 1 U.	71.5	36.8	23.5	33.9	23.2	34.0	28.3	34.8	36.6	35.2	−33
K	Morphine Sulfate 25 mg. & Insulin 1 U.	70.4	37.2	25.2	33.0	17.5	32.8	26.1	33.4	31.6	35.6	−37, 1 death during hr. 2
L	Morphine Sulfate 50 mg. & Insulin 1 U.	70.5	37.8	22.6	31.9	16.6	31.4	15.7	31.7	18.5	33.0	*1, −40

D), barbital sodium (Groups E and F), or by morphine sulfate (Groups J, K and L). According to the averages, meperidine hydrochloride, at a 15 mg. dose level (Group G, Table 3) had no effect; 30 mg. (Group H) produced a slight decrease and 50 mg. (Group I) caused a slight increase. Considerable individual variations also were noticed.

Insulin (Table 4, Group A), as would be expected, depressed the blood sugar level but not as greatly as under the conditions in Table 1. The administration of pentobarbital sodium (Groups B and C) or evipal sodium (Group D) slightly decreased the hypoglycemic effect of insulin. Barbital sodium in a dose of 100 mg. (Group E) slightly decreased the hypoglycemic response to insulin but the 200 mg. dose (Group F) had no effect. Meperidine hydrochloride, 15 mg. (Group G) had no effect, but 30 mg. (Group H) increased and 50 mg. (Group I) decreased the hypoglycemic action of insulin. Morphine sulfate, 12 mg. (Group J) did not alter the hypoglycemic action of insulin whereas with 25 and 50 mg. (Groups K and L) this effect was intensified slightly.

The rectal temperatures were depressed by all drugs used, with the greatest effect being produced by meperidine hydrochloride and morphine sulfate (except the 12 mg. dose which is a minimal analgesic dose) (Table 3, Groups H, I, K, and L). The combination of pentobarbital sodium or barbital sodium with insulin (Table 4) produced hypothermic effects which were in most cases less than a summation effect; but with evipal sodium, meperidine hydrochloride or morphine sulfate with insulin, the hypothermic effects were slightly greater than summation in most instances.

DISCUSSION

The average control fasting blood sugar values (Tables 1, 3 and 4) observed in this study are in agreement with those reported by certain previous investigators (Greisheimer et al., 1935; Dotti and Hrubetz, 1936; Cole and Harned, 1938). These values for blood sugar may seem low but it should be recalled that the methods used do not show the non-fermentable reducing fraction. The variations observed were comparable to those previously observed by Greisheimer et al. (1935) and by Dotti and Hrubetz (1936). The blood sugars of the two normal groups which were kept at different temperatures (A, Table 1 and A, Table 3) do not differ significantly. It was deemed advisable however, to obtain separate normals as the tail of the rat has been reported to be of importance in temperature regulation (Knoppers, 1942) and this factor might influence the level of the blood sugar.

Morphine sulfate in small doses (12 mg.) did not cause any change in the blood sugar level of the rat (Group B, Table 1 and Group J, Table 3). In other species (cat, dog, and rabbit) morphine at this dose level produces hyperglycemia (Ross, 1918; Stewart and Rogoff, 1922a; Morrison, 1947). The effect of larger doses (25 and 50 mg.) is

to depress the blood sugar level in some instances and several of the rats died, presumably from depression of the respiratory center.[6] Since the peripheral tissues are constantly removing sugar from the blood (Soskin and Levine, 1946a) the glucose content of the tail blood of a moribund rat with a poor circulation from large doses of morphine could be expected to be low. Ingle (1945) in his review has reported that in fasted rats, rapidly induced anoxia causes an initial hyperglycemia which is followed by a hypoglycemia. Van Middlesworth et al. (1944) have found that anoxia in fasted rats causes hypoglycemia. Considering these reports, the depressed respiration (with a concomitant anoxia) due to large doses of morphine sulfate, could be a factor in the hypoglycemias observed in the present experiments. In some individual instances a few of the blood sugars were high enough (Table 1, Groups C and D, Table 3, Groups K and L) to account for the high averages in spite of some very low values. The urine of the rats dying from overdosage was tested for glucose in nearly every instance and found to be negative, evidently the hyperglycemia in these animals had not been high enough to exceed the renal threshold. From their results Feldman et al. (1940) believe that morphine acts on both sympathetic and parasympathetic centers to produce either hyperglycemia or hypoglycemia. This explanation may be applicable to the variations observed in the present investigation.

Barbital was reported by Ellís and Barlow (1924) to produce hypoglycemia whereas Weiss (1926) and La Barre and Vesselovsky (1942) have reported that barbital causes hyperglycemia. In the present study, barbital sodium in the rat (Tables 1 and 3) tended to lower the blood sugar in most instances. With a dose of 200 mg., barbital sodium depressed the rats markedly, causing death in five of twenty animals (Group F, Tables 1 and 3).

Evipal sodium does not alter the blood sugar level of man (Jarman and Abel, 1933) or of the rabbit and guinea pig (Kennedy and Narayana, 1934). In the present study using the rat, evipal also showed no effect on the blood sugar level.

Pentobarbital causes hyperglycemia in dogs (Hemingway et al., 1934) and cats (Smith et al., 1948) but in mice Heistand et al., 1947) and rabbits (Blackberg and Hrubetz, 1936) hypoglycemia is produced. In the present investigation, no significant changes due to pentobarbital were noted in the average blood sugar level in the rat.

Morphine sulfate in small doses (12 mg. Tables 1 and 4) does not seem to have any decided effect on the hypoglycemic effect of insulin. However, there were no deaths in Group H, Table 1, as compared with three deaths in Group G, Table 1, which received only insulin. That morphine in this dosage had an antagonistic action to the hypoglycemic effect of insulin was reported by the author (Larson, 1945) in a preliminary communication after doing most of the experiments reported in Table 1 but not those of Table 4. After repeating some of the

early experiments and having completed those of Table 4, the author feels that his previous conclusion was not completely warranted. At a higher dose level (25 and 50 mg.), morphine sulfate was found in the present study to have an additive effect on the hypoglycemia produced by insulin. This is shown by the low blood sugars, especially at the third and fourth hours as compared with those of the animals receiving insulin only. This retardation in the recovery of the blood sugar level by morphine is comparable to the effects of phenobarbital sodium observed by Johlin (1947) and probably is due to depression of the antagonistic factors (Bouckaret and Duve, 1947) to hypoglycemia. In these four groups receiving 25 and 50 mg. morphine sulfate, ten animals died and two were prostrated. This accentuated toxicity of morphine-insulin combination might have been expected since Seevers and Shideman (1941) reported that morphine decreased the oxygen uptake by the cerebrum. Quastel (1939) has concluded that narcotics produce an anoxemia in those parts of the central nervous system which they affect and this would interfere with oxidation of glucose (already present in a lowered concentration) which is indispensable for the maintenance of the functional integrity of the nervous system (Soskin and Levine, 1946b). That morphine sulfate, causing an anoxia by respiratory depression, could increase the hypoglycemic effect of insulin at hours 2, 3 and 4 might have been expected since Gellhorn and Packer (1940) have reported that short periods of anoxia act antagonistically to insulin whereas longer periods (2 hrs.) aggravated the hypoglycemia. Similar conclusions have been drawn by Van Liere (1942). This depression of respiration by morphine might intensify the respiratory action of insulin since Voegtlin et al. (1924) have shown that insulin in the rat causes pulmonary edema and may cause death by respiratory failure. Lundback (1944) later has reported that insulin lowers the sensitivity of the respiratory center.

Following the injection of barbital sodium (Groups K and L, Table 1, and Groups E and F, Table 4), pentobarbital sodium (Groups E and F, Table 4), pentobarbital sodium (Groups B and C, Table 4) or evipal sodium (Group D, Table 4), the hypoglycemic effect of insulin in nearly every instance was the same or slightly less than during the corresponding periods with insulin alone. It also should be noted that in the group (Group K, Table 1) receiving 100 mg. barbital sodium plus insulin there were no deaths whereas in the group (Group G, Table 1) which received the same dose of insulin without the barbiturate, 3 of the 10 animals died. This protective action of barbital sod in the rat is similar to the results of Jackson (1931) who found that barbital sodium in rabbits tended to suppress convulsions and deaths due to insulin. Confirmatory evidence of this anticonvulsive action of barbital sodium has been reported (Nakajima, 1938; Andreeva, 1947) and also denied (Okumura, 1939). The depression of metabolism by large doses of barbiturates (Goodman and Gilman,

1941a) would require less glucose for combustion, thereby permitting a higher blood level. This would also be consistent with the view that narcotics in general inhibit oxidation of glucose in the brain (Quastel, 1939) and also the findings of Handley et al. (1941) that pentobarbital reduces the glucose uptake by the brain. In rabbits, Jackson (1931), who used doses varying from 25 to 150 mg. of barbital sodium, found that the 100 mg. dose had the maximum antihypoglycemic effect toward insulin. Since anesthesia with the barbiturates does not alter the insulin content of the pancreas (Haist, 1944), it would seem that any blood sugar effects are extra-pancreatic.

Meperidine hydrochloride, in rabbits receiving a 10 mg. dose, has no effect on the blood sugar level but 20 mg. causes a slight decrease (Barlow, 1947). The action of meperidine hydrochloride in slightly increasing the hypoglycemic action of insulin (Group H, Table 4) might be expected from the action in the normal rat (Group H, Table 3) and also the tendency for the antihypoglycemic action (Group I, Table 4) could be anticipated from the slightly increased blood sugar level (Group I, Table 3). This action of meperidine can be compared to the biphasic action of morphine on the central nervous system (Hazelton and Koppanyi, 1941). The effect of meperidine hydrochloride in producing variations, both hypoglycemia and hyperglycemia in the blood sugar level, was similar to that of morphine sulfate. Since meperidine does not influence the uptake of oxygen by the brain (Elliott et al., 1947), these effects cannot be explained in a manner similar to that proposed previously for the action of morphine. The S-shaped tail reaction, as reported previously by Gruber et al. (1941) for meperidine, was observed.

The normal rectal temperature of the rat has been reported variously from 36.7° C. (Herrington, 1940) to 37.27° C. (Hill, 1947). The temperatures observed in this investigation (Tables 2, 3 and 4) agree with the latter report. The general statement (Goodman and Gilman, 1941b: Sollmann, 1948a) that morphine decreases the body temperature is true of most reports except in the cat (Stewart and Rogoff, 1922b) and the rat (Herrmann, 1941) in which hyperthermia has been reported. In the present investigation from the average temperatures of 40 animals, there was a fall in body temperature produced by the 25 or 50 mg. dose of morphine sulfate (Tables 2 and 3). Morphine sulfate showed a greater toxicity due to the higher environmental temperature (deaths in Tables 1 and 2 compared with Table 3). Chen et al. (1943) have reported an increase in toxicity for certain other drugs when the environmental temperature was increased.

Barbital sodium, pentobarbital sodium and evipal sodium have been reported by previous investigators (Kennedy and Narayana, 1934; Sollmann, 1948b) to lower the body temperature and this effect was observed in the present experiments (Table 3). Meperidine hydro-

chloride (Table 3) also lowered the body temperature of the rat, presumably by central depression.

The body temperature of mice was decreased by insulin (Dudley et al., 1923) and this effect in the rat was observed to a slight degree (Table 4) in this investigation though Visscher and Green (1925) have reported no uniformity of change in rabbits. As previously reported (Voegtlin and Dunn, 1923: Chen et al., 1943; Johlin, 1944), insulin in the present experiments caused a greater hypoglycemic effect in the warmer environment (Group G, Table 1; Group A, Table 4). If Fuhrman's (1946) hypothesis that increasing the temperature increases the enzymatic inactivation of drugs, it would seem that the inactivation of insulin by the tissues is not enzymatic in nature but that the action of insulin is enzymatic as suggested by Soskin and Levine (1946c).

No adequate explanation can be offered for an effect greater than summation on temperature depression of evipal sodium, meperidine hydrochloride or morphine sulfate in combination with insulin nor for an effect less than summation when insulin was combined with pentobarbital sodium or barbital sodium.

CONCLUSIONS

Morphine sulfate in small doses (12 mg. per kg.) has no effect on the blood sugar level but larger doses (25 or 50 mg.) may produce either a definite hypoglycemia or hyperglycemia.

Morphine sulfate in small doses (12 mg. per kg.) does not affect the hypoglycemic action of insulin but larger does (25 or 50 mg.) prolong the hypoglycemic action.

Barbital sodium (100 to 200 mg. per kg.), pentobarbital sodium (25 mg. or 35 mg. per kg.) or evipal sodium (100 mg. per kg.) do not affect the blood sugar level of the normal animal but slightly decrease the hypoglycemic effect of insulin.

Meperidine hydrochloride (demerol hydrochloride), 15 mg. per kg. had no effect on either the blood sugar level of the normal animal or of the animal receiving insulin. Meperidine hydrochloride 30 mg. per kg., slightly depressed the blood sugar level of the normal rat and also of the animals receiving insulin. Meperidine hydrochloride, 50 mg. per kg., increased the blood sugar level of both the normal animals and of those receiving insulin.

All the drugs used: barbital sodium, pentobarbital sodium, evipal sodium, morphine sulfate, meperidine hydrochloride or insulin, depressed the rectal temperature.

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THE EFFECT OF ADRENALECTOMY ON THE RENAL TUBULAR REABSORPTION OF WATER IN THE RAT[1]

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IT IS well known that the adrenal insufficient animal excretes a given dose of water at a slower rate than the normal (Gaunt, 1946). The purpose of the present paper is to present data which indicate that the basis for this deficiency in water excretion is an increased reabsorption of water by the renal tubules in the absence of the adrenal cortical hormones.

MATERIALS AND METHODS

Three groups of Sprague-Dawley female rats were used; 12 ad libitum fed controls, 11 adrenalectomized and 6 pair-fed controls. The rats weighed from 200-250 grams and were fed GLF "calf meal." The pair-fed controls were started a day following the operated animals, and received only as much ration as the latter had eaten in the previous 24 hr. period. Thus any effects of post-operative inanition were eliminated in interpreting the results. Following adrenalectomy the operated rats were given 1% sodium chloride to drink and were not used for at least two weeks, by which time they had completely recovered from the operation. Daily records of body weight were kept on all animals. The completeness of adrenalectomy was established by the death of all animals within one week after withdrawal of salt.

The creatinine clearance was used as a measure of the rate of glomerular filtration, the technique being modified from Friedman, Polley and Friedman (1947) and Stevenson (1947). A single subcutaneous injection of 5 per cent creatinine was given in amounts of 0.5 ml. per 100 grams of rat. It was found that with this procedure the plasma creatinine at the end of 30 minutes was about 20 mgm. % and remained constant at that level over the following 30 minutes. This fact justified the use of a terminal blood sample in the determination of the creatinine clearance and eliminated the depressive effects of tail cutting as pointed out by Lippman (1948). Accordingly after a 12 hr. fast the rats were given creatinine by subcutaneous injection and 5 ml. water by stomach tube. Thirty minutes later the bladder was emptied manually by suprapubic pressure and a 30 minute collection period begun. Urine collection was made with the rats in large funnels. At the end of the period the bladder was emptied into the funnel, the sides of which were washed with distilled water and the washings added to the original urine. Plasma and

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urine samples were then analyzed for creatinine content. The creatinine clearances obtained by this method agree more closely with recently published values (Lippman, 1948; Corcoran, Masson, Renting and Page, 1948) than with previous observations (Friedman and Livingston, 1942; Dicker and Heller, 1945) which were lower.

RESULTS

In Table 1 are presented the mean values for the several observations made on each group of animals. In order for two groups of observations to be significantly different, the difference between their means had to be greater than three times the standard error of the difference. By this criterion the rates of glomerular filtration were not different in the three groups, but the urine flow of the adrenalectomized group was significantly lower than that of either control group.

TABLE 1. SUMMARY OF EXPERIMENTS ON THE RATE OF GLOMERULAR FILTRATION AND URINE FLOW IN NORMAL AND ADRENALECTOMIZED, SALT-MAINTAINED RATS

Groups	Number of animals	Body Weight	Glomerular filtration rate	Urine Flow
		Grams	ml./100cm.²/min.	ml./100cm.²/min.
Ad libitum fed controls	12	250 ± 3.30	.513 ± .0300	.0111 ± .00416
Adrenalectomized	11	251 ± 4.80	.490 ± .0340	.00620 ± .000734
Pair fed controls	6	245 ± 4.80	.467 ± .0180	.0135 ± .00116

Figures are the mean of the observations in each group with the standard error. Surface area determined according to the Meeh formula; $SA = 11.23 \times \text{weight}^{2/3}$

DISCUSSION

These data indicate that the adrenalectomized rat maintained on salt has a normal rate of glomerular filtration, and a decreased rate of water excretion which results from an increased rate of tubular reabsorption of water. Birnie, Jenkins, Eversole and Gaunt, (1948) have observed that the blood of adrenalectomized rats contains larger quantities of antidiuretic substance than that of normals. Since it is known that antidiuretic hormone acts to accelerate the tubular reabsorption of water, their observation might constitute an adequate explanation for the increased rate of tubular reabsorption of water reported here. In their paper these authors present a discussion of mechanisms involved.

SUMMARY

Experiments on rats have shown that the decreased capacity of the adrenal insufficient animal to excrete water is not necessarily due to a decreased rate of glomerular filtration but is rather the result of an increased rate of renal tubular reabsorption of water.

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HYDROLYSIS OF CONJUGATED SULFATES OF ESTROGENS BY COMMERCIAL ENZYME PREPARATION OF ASPERGILLUS ORYZAE

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MOST of the steroids in freshly voided urine occur as water-soluble esters of either sulfuric or glucuronic acid. In the past, determination of estrogens in urine has been based upon acid hydrolysis of the conjugated estrogens with subsequent extraction of the free estrogens by organic solvents. Van Bruggen, (1948) has recently re-evaluated various methods of acid hydrolysis and estimated the extent of destruction of estrogens during the hydrolysis. Hydrolysis of the conjugated estrogens in human urine by B. coli has been reported by Patterson (1937) to be 75 per cent as efficient as acid hydrolysis.

It has long been known that preparations made from aspergillus oryzae contained a sulfatase. Neuberg et al. (1923a, 1923b, 1926) and Noguchi (1923) described the sulfatase activity of such extracts and claimed that only aromatic ethereal sulfates were subject to hydrolysis by this enzyme. Dzialoszynski (1947) used such an enzyme extract, Clarase, to determine the ethereal sulfate content of equine urine and concluded that this enzyme was inhibited by the urine substrate. We have found, however, complete hydrolysis of the conjugated estrogens in mares' urine using two different commercial enzyme preparations.

Butenandt and Hofstetter (1939) employed Takadiastase as a source of phenolsulfatase for hydrolysis of their synthetic estrone sulfate in order to prove its ester linkage, but no one has reported successful enzyme hydrolysis of urinary conjugated estrogens. We have studied the hydrolysis, by phenolsulfatase preparations, of the conjugated estrogens in pregnant mares' urine and in semi-purified extracts of the conjugated sulfates from such urine. Higher yields of estrogens were obtained by enzyme hydrolysis than by acid hydrolysis. Since enzyme hydrolysis is more specific than acid hydrolysis, much purer extracts are obtained.

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MATERIALS AND METHODS

Mylase and Mylase P,[1] water-soluble enzyme preparations obtained from aspergillus oryzae, were used as a source of phenolsulfatase.

The urine substrate was prepared by dissolving dried urine[2] of pregnant mares in water. A stable source of comparable urine was thus always available.

The crude conjugated sulfate preparation used was prepared from the above powdered urine as follows: Four extractions were made using 80–90% acetone (2 cc./gm. of urine powder). A two phase separation occurs which makes decantation easy. The acetone extracts were evaporated to dryness in vacuo and the residue suspended in a minimal volume of water. The aqueous suspension was adjusted to pH 3.0 and shaken twice with one-half volume of ether. Ether clears the solution and removes free estrogens, organic acids and other soluble compounds. The ether-washed aqueous solution was adjusted at once to pH 7.0 and extracted four times with one-half volumes of normal butanol. The butanol was removed in vacuo using a Megavac pump and a bath temperature of 50–70°C. The residue from the evaporated butanol extracts was dissolved in a small volume of water and lyophilized. The dried powder (Preparation M) was found to contain 27.7% sulfate, 9.23% sulphur, 4.38% ash and 1.3% of estrogen expressed as estrone. This preparation, like others similarly prepared, gives no precipitate with barium chloride until after slight heating in a solution more acid than pH 4.0.

All enzyme digestions were carried out at pH 6.0 to 6.4 and at a temperature of 50°C., conditions described by Abbott (1947) as optimum for phenolsulfatase. Digestions were carried out in individual tubes (usually 15 ml. centrifuge tubes) for each time interval in the time studies. At the end of the incubation period the digest was shaken with toluene to extract the free estrogens. The tubes were centrifuged and the toluene phase was removed and washed with 10 per cent sodium carbonate. Only one extraction with toluene was made. Fluorimetric determinations were made directly on the toluene phase.

The conjugates in urine powder were acid hydrolyzed by adjusting an aqueous solution of the powder to about pH 1.0 with hydrochloric acid, covering it with a volume of toluene equal to that of the solution and refluxing for 2 to 3 hours. Higher values are obtained when toluene is present during the hydrolysis than when no solvent is used. In the case of Preparation M, hydrolysis at pH 1.0 for 45 minutes was found to yield maximum estrogen values.

The estrogen content of the toluene extracts was determined by the fluorimetric method of Bates and Cohen (1947) using a 4200 Å interference filter as a lamp filter and a 5200 Å interference filter plus a 3387 Corning glass filter as photocell filters. All values were determined using estrone as a standard and are expressed as micrograms of estrone.

EXPERIMENTAL

In order to determine the quantity of Mylase P required for com-

[1] Wallerstein Laboratories, New York City. In preliminary tests we used successfully a diastatic preparation from aspergillus oryzae obtained from Paul Lewis Laboratories, Milwaukee, Wisconsin.

[2] From Dr. A. D. Odell, Steroid Laboratories Ltd., Montreal, Canada.

plete hydrolysis of the conjugated estrogens, studies were made using the conjugated sulfate Preparation M as the substrate and varying the concentration of enzyme and the time of digestion. The results are presented in Figure 1. It is evident that with a 2 per cent enzyme concentration and after 8 hours digestion, the maximum amount of free estrogen obtained slightly exceeded that obtained with acid hydrolysis. During digestion there was apparently some destruction of estrogens as a result of which the maximum amount of free estrogen,

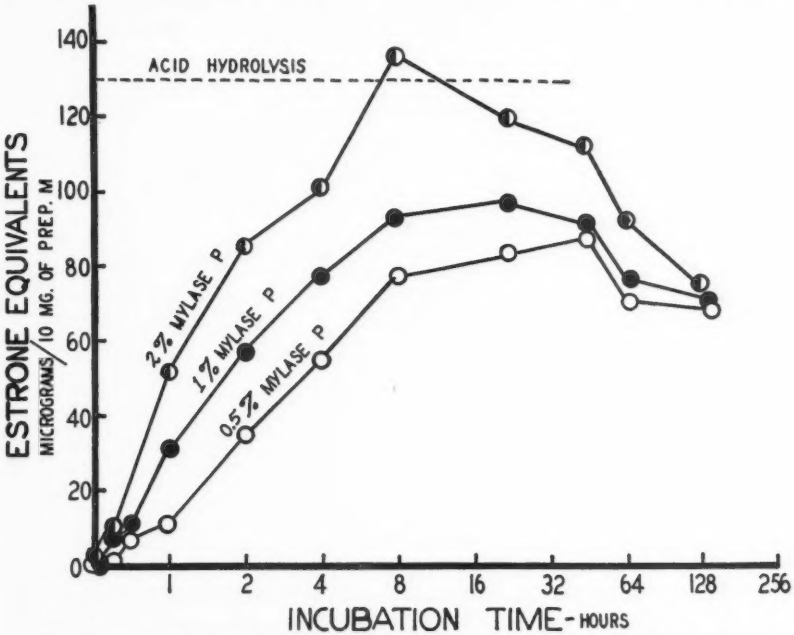

FIG. 1. Rate of enzyme hydrolysis of conjugated estrogen as a function of enzyme concentration at pH 6.0 and at 50°C. Ordinate indicates the amount of free toluene-soluble estrogen. 100 mg. of Preparation M. was dissolved in 5 ml. of water with 100, 50 or 25 mg. of Mylase P respectively in each tube. Controls containing boiled enzyme showed no hydrolysis even after 144 hours.

obtained, with the lower concentrations of enzyme, was only about 70 per cent of that with acid hydrolysis. Controls, consisting of substrate with no enzyme and of substrate plus heat-inactivated enzyme, show no hydrolysis even after 144 hours.

It may be pointed out here that, in order to get as much hydrolysis in 24 hours with enzyme as in 1 hour with acid, the weight of Mylase P used has to be equal to that of Preparation M which contained only 1.3 per cent estrogen. Hence, the phenolsulfatase content of Mylase P is probably very small.

Because the destruction of estrogenic material by Mylase P might

be due to oxidative changes, the series of tests shown in Figure 2 were done. A thick layer of toluene was placed on top of the aqueous digest. This served two purposes: one was to exclude air and the other was to remove the free estrogen from destructive action of the enzymes in the aqueous phase. It is obvious, from the data shown in Figure 2, that the toluene layer in no way inhibits the rate of digestion but does inhibit the destructive action. In addition, the total amount of estrogen extracted by the toluene is about 20 per cent greater than that obtained by acid hydrolysis. The hydrolysis was not complete until

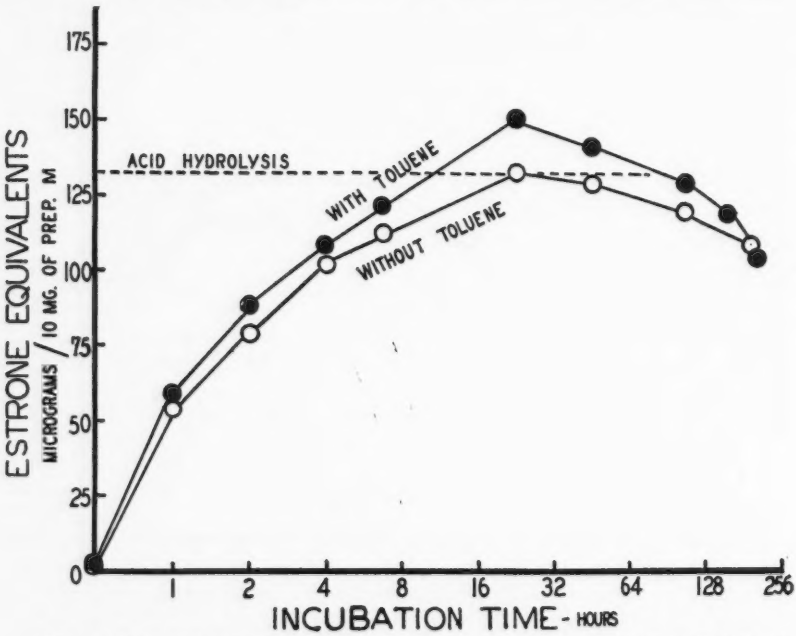

FIG. 2. Rate and extent of hydrolysis of conjugated estrogen with and without toluene at pH 6.0 and 50°C. Ordinate indicates the amount of free toluene-soluble estrogen. 10 mg. of Preparation M and 10 mg. of Mylase P were dissolved in 5 ml. of water in each tube.

24 hours. On longer standing, evidence of destruction was again obtained.

Further indication of the oxidative destruction of the steroid by the enzyme was obtained by subjecting to enzyme digestion for 18 hours two tubes of a solution of pregnant mares' urine powder containing 200 micrograms of estrone equivalent by acid hydrolysis. Both tubes were overlaid with toluene and treated similarly except that air was bubbled through one. The estrogen extracted by toluene from the solution in the tube incubated without air was equivalent to 220 micrograms of estrone. Only 120 micrograms, on the other hand,

were extracted by toluene from the tube which had air passed through it.

In Figure 3 are shown the results obtained upon incubation of a freshly prepared solution of pregnant mares' urine powder with Mylase P, both with and without an overlay of toluene. Once again, the destructive process is evident in the tubes incubated without toluene, while some inhibition of this phenomenon is observed when the tubes are kept covered with toluene. In addition, the total amount of estrogen extracted by the toluene is about 30 per cent greater than that obtained by acid hydrolysis. Controls, consisting of substrate with no

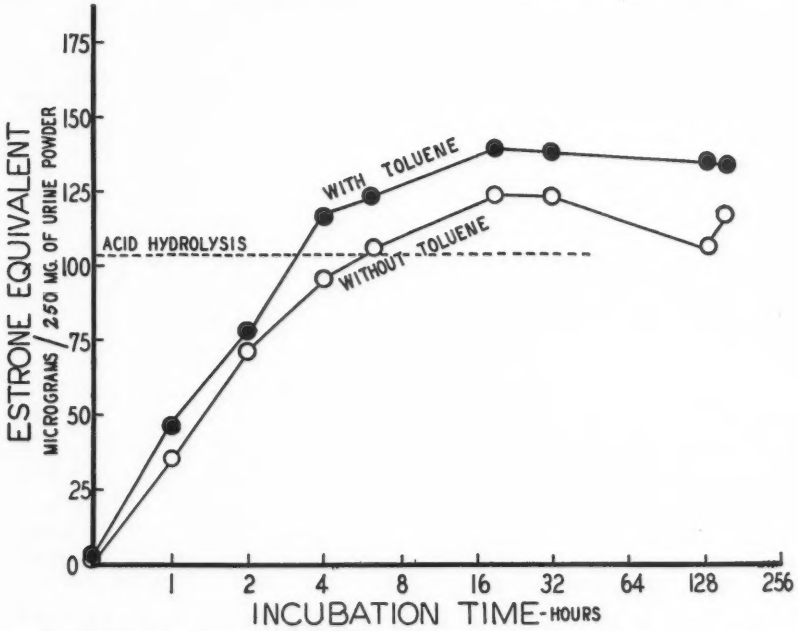

FIG. 3. Rate and extent of hydrolysis of conjugated estrogen with and without toluene at pH 6.4 and 50°C. Ordinate indicates the amount of free toluene-soluble estrogen. 250 mg. of urine powder and 40 mg. of Mylase P were dissolved in 5 ml. of water in each tube.

enzyme and of substrate plus heat-inactivated enzyme, gave no hydrolysis even after 144 hours.

The increased rate of destruction by Mylase P of the estrogens in the conjugate Preparation M, as compared to the rate of destruction in the reconstituted urine, may be due to the removal of protective substances during the purification of Preparation M since 98 per cent of the solids present in the urine were removed during its preparation. Similar observations have been made by Smith and Smith (1941) who found that crystalline estrone in aqueous solution will have 50 per

cent of its potency destroyed by acid hydrolysis at 100°C. for 20 minutes, while in the presence of urine such destruction does not occur.

Having established the effectiveness of Mylase P and the optimum hydrolyzing conditions, larger amounts of substrate were subjected to enzyme hydrolysis and then fractionated. Fractionation was carried out according to the scheme shown in Figure 4.

FIGURE 4

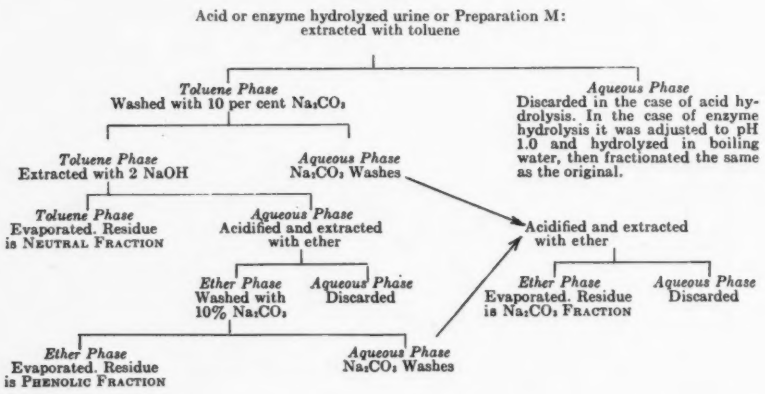
Acid or enzyme hydrolyzed urine or Preparation M: extracted with toluene
Toluene Phase
Washed with 10 per cent Na2CO3
Aqueous Phase
Discarded in the case of acid hydrolysis. In the case of enzyme hydrolysis it was adjusted to pH 1.0 and hydrolyzed in boiling water, then fractionated the same as the original.
Toluene Phase
Extracted with 2 NaOH
Aqueous Phase
Na2CO3 Washes
Toluene Phase
Evaporated. Residue is NEUTRAL FRACTION
Aqueous Phase
Acidified and extracted with ether
Acidified and extracted with ether
Ether Phase
Washed with 10% Na2CO3
Aqueous Phase
Discarded
Ether Phase
Evaporated. Residue is Na2CO3 FRACTION
Aqueous Phase
Discarded
Ether Phase
Evaporated. Residue is PHENOLIC FRACTION
Aqueous Phase
Na2CO3 Washes

Table 1 is a compilation of the results obtained upon the subjection of 1 gram of conjugated sulfate Preparation M to enzyme and acid hydrolysis in the presence of toluene. The superiority of enzyme hydrolysis over acid hydrolysis is shown; 1) by higher yield of estrogen in the phenolic fraction with lower weight of non-estrogenic material, which means greater purity, 2) by much less color at all stages of fractionation and 3) by the crystalline nature of the phenolic fraction from enzyme hydrolysis.

During acid hydrolysis, many toluene-soluble impurities are formed. This is indicated by the fact that acid hydrolysis of the aqueous residue remaining after toluene extraction of the enzyme digest (Line 3, Table 1) yields fractions which, when added to the fractions obtained after enzyme digestion (Line 2, Table 1) give totals approximately those obtained after only acid hydrolysis of Preparation M (Line 1, Table 1). The phenolic fraction is an exception.

Table 2 presents data obtained in subjecting a freshly prepared solution of pregnant mares' urine powder to enzyme hydrolysis or acid hydrolysis. Again the efficiency of enzyme hydrolysis is very well illustrated by first, a greater yield of estrogens than the yield obtained by acid hydrolysis, and second, by the smaller amount of impurities obtained. In the phenolic fraction, isolated after enzyme hydrolysis, 20 per cent of the weight is estrogenic material while in the

TABLE 1. COMPARISON OF ENZYME AND ACID HYDROLYSIS OF 1 GRAM OF CONJUGATED ESTROGEN PREPARATION M CONTAINING AN ESTIMATED 13 MG. OF ESTROGEN. THE AQUEOUS PHASE REMAINING AFTER ENZYME HYDROLYSIS (LINE 2) WAS ACID HYDROLYZED (LINE 3). A LAYER OF TOLUENE WAS PRESENT DURING ALL DIGESTIONS

Experimental Procedure	Na_2CO_3 Fraction	Neutral Fraction			Phenolic Fraction		
	Weight	Weight	Optical Density*	Estrogen Content	Weight	Optical Density*	Estrogen Content
	mg.	mg.		mg.	mg.		mg.
Acid Hydrolysis: 1. 1 gm. conjugate M in 50 ml. H_2O at pH 1.3 Refluxed 45 minutes	32.0	18.2	1.3	0.45	137.0 (tar)	0.73	11.9
Enzyme Hydrolysis: 2. 1 gm. conjugate M 1 gm. Mylase P 50 ml. H_2O at pH 6.0 20 hrs. at 50° C.	9.0	8.0	0.17	0.23	39.0 (crystals)	0.47	13.0
Acid Hydrolysis: 3. Aqueous phase from (2) Adjusted to pH 1.2 Refluxed 50 minutes	21.2	12.0	1.5	0.10	9.5 (tar)	0.56	0.25

* Optical density at 420 mu. of a solution of the entire fraction in 25 ml.of ethanol.

TABLE 2. COMPARISON OF ENZYME AND ACID HYDROLYSIS OF ONE KILO OF URINE POWDER EQUIVALENT TO ABOUT 20 LITERS OF PREGNANT MARES' URINE. THE AQUEOUS PHASE REMAINING AFTER ENZYME HYDROLYSIS (LINE 2) WAS ACID HYDROLYZED (LINE 3). A LAYER OF TOLUENE WAS PRESENT DURING ALL DIGESTIONS.

Experimental Procedure	Na_2CO_3 Fraction		Neutral Fraction		Phenolic Fraction	
	Weight	Estrogen Content	Weight	Estrogen Content	Weight	Estrogen Content
	gm.	mg.	gm.	mg.	gm.	mg.
Acid Hydrolysis: 1. 1 kilo of urine powder in 10 liters of H_2O at pH 1.0 Reflux 3 hours	79.0	0	2.3	43.0	12.1	540.0
Enzyme Hydrolysis: 2. 1 kilo of urine powder with 100 gm. Mylase P in 10 liters of H_2O at pH 6.2 18 hours at 50°C.	1.5	0	0.36	13.3	3.25	683.0
Acid Hydrolysis: 3. Aqueous phase from (2). Adjusted to pH 1.0 Refluxed 3 hours	73.0	0	2.80	8.75	5.5	35.0

fraction isolated after acid hydrolysis, only 5 per cent of the weight is estrogenic material. The weights of the carbonate-soluble and neutral fractions show a still greater difference between enzyme and acid hydrolysis.

Preliminary experiments with human pregnancy urine (5th month gestation) indicate that a portion of the estrogens, presumably sulfates, is hydrolyzed by Mylase P.

CONCLUSIONS

From the data in Table 1 and 2 it may be concluded that the hydrolyzing activity of the enzyme is largely due to a phenolsulfatase because the weight of the neutral fraction obtained after enzyme hydrolysis relative to that obtained after acid hydrolysis, is only about 44 per cent and 16 per cent respectively. This indicates that part of the conjugated sulfates, presumably non-phenolic conjugates, is not hydrolyzed by the enzyme. Hence, Mylase P does not contain a general sulfatase.

The phenolsulfatase specificity of Mylase P is further indicated by the fact that Mylase P does not hydrolyze phenolphthalein glucuronide. Hence, it does not contain a general phenolesterase (Fishman, 1948).

Our data support the conclusions of Noguchi (1923) that aspergillus oryzae extracts contain a specific phenolsulfatase.

SUMMARY

Mylase P, a commercial enzyme preparation from aspergillus oryzae, was found to hydrolyze the conjugated estrogens present in the urine of pregnant mares. After 24 hours digestion at pH 6.0 and 50°C. under a layer of toluene with relatively large amounts of Mylase P, about 20 per cent higher yields of estrogens were obtained than after acid hydrolysis.

The phenolic fraction isolated after enzyme hydrolysis contained a much higher percentage of estrogens than that isolated after acid hydrolysis.

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PRELIMINARY OBSERVATIONS ON THE ANTIDIURETIC SUBSTANCE RECOVERED FROM THE URINES OF PATIENTS WITH CIRRHOSIS OF THE LIVER

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In 1945 Ralli et al. reported an increase in the amount of antidiuretic substance present in the urines of patients with cirrhosis of the liver and ascites. Recently Drill and Frame (1948) have confirmed this observation. The presence of increased amounts of an antidiuretic substance in the urine of patients with water retention is of interest because of the possible relationship that the substance in urine bears to the antidiuretic hormone of the posterior pituitary and because of the role of this hormone in urine flow (Wesson, 1948) and therefore in water balance (Gilman and Goodman, 1936). Verney (1946, 1947) and Pickford (1945) have shown that the antidiuretic hormone regulates the rate of urine flow, and Verney (1947, 1948) has described the mechanism for the control of the secretion of the hormone. Although much is known of the properties of the hormones of the posterior pituitary gland and the mechanism of their elaboration, many gaps in our knowledge of these hormones remain, and this is especially true of the antidiuretic hormone (Chow, 1944; Van Dyke, 1943).

In an effort to study the role of the antidiuretic substance in the formation of ascites in patients with cirrhosis of the liver, it was thought advisable to study the nature of the antidiuretic substance in urine. This report deals with the initial phases of the processing of the urines from patients with cirrhosis of the liver.

PROCEDURE AND RESULTS

Urines from patients with cirrhosis of the liver in various stages of the disease were collected and pooled. Toluene and sufficient 25% acetic acid to bring the urine to pH 4 were used as preservatives. The urine was processed in batches of from 1 to 5 liters.

Dialysis. Each liter of urine was divided into 200 cc. aliquots, and each aliquot was contained in a membrane made of Visking cellulose

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sausage casing (36/32 Nojax). Five of these aliquots were dialyzed against 5 gallons of distilled water in an earthenware crock for 5 days. The dialyzing water was changed once or twice a day for the first 3 days and daily for the last 2 days. Both the urine and the water were protected against decomposition with toluene. It is probable, in the light of subsequent experiments, that a considerable loss of the antidiuretic substance occurred during this prolonged dialysis. This point will be discussed in more detail later.

Concentration of the Dialysate. After dialysis each aliquot, in the same casing in which it had been dialyzed, was concentrated by suspension in front of a fan. This required approximately 24 hours.

In order to establish the degree of concentration necessary to obtain an antidiuretic effect after prolonged dialysis, samples from individual patients were concentrated to different extents ranging from 1/8th to 1/68th of the original volume. They were then tested for antidiuretic activity by injection into hydrated rats, as previously described (Ralli et al., 1945). The results, which are shown in Table 1, indicate that urine which has been dialyzed for long periods must be concentrated at least to 1/40th of its original volume before a positive antidiuretic test can be obtained. Samples "C" and "E" were tested at 2 different levels of concentration, and only when the concentration was about 40 times that of the original urine was a positive antidiuretic test obtained. In the subsequent processing, dialyzed pooled urines were therefore concentrated to 1/40th of their original volumes.

TABLE 1. RELATIONSHIP OF THE DEGREE OF CONCENTRATION OF DIALYZED URINE TO THE BIOLOGICAL TEST FOR THE ANTIDIURETIC SUBSTANCE

Urine Sample	Initial Volum ml.	Concentrated Volume ml.	Concentration Factor	Biological Test
H	2,820	350	8	Negative
J	700	42	17	Negative
A	4,475	208	22	Positive
F	1,900	73	26	Negative
D	7,180	200	36	Negative
G	4,100	108	38	Negative
I	966	25	39	Positive
M	2,300	55	42	Positive
L	1,700	35	49	Positive
B	5,925	100	59	Positive
K	4,850	80	61	Positive
C	5,440	300	18	Negative
C	5,440	80	68	Positive
E	1,820	175	10	Negative
E	2,000	50	40	Positive

The concentrated urine was then filtered in the cold, using a coarse filter paper. The filtrate was tested biologically for its antidiuretic activity and was found to contain significant amounts of antidiuretic substance.

Chromatographic Treatment. The filtrate was then chromatographed, using a column of Permutit prepared according to Folin. Potts and Gallagher (1944) have shown that the pressor principle of Pitressin can be separated from the oxytocic principles by chromatographic procedures using Permutit. We did no analyses for the pressor or oxytocic principles, but rather have studied the behavior of the antidiuretic substance on the Permutit column and the eluation of this substance by 5% NaCl-1 M acetic acid.

The columns were 42.5 cm. long (with an inside diameter of 1.5 cm.) and contained 35 gm. of Permutit. The bottom of the tube contained a glass wool plug and a rubber stopper through which a small piece of capillary tubing was inserted. The Permutit was packed into the column and then washed with 300 cc. of distilled water, using suction. The material to be chromatographed was delivered to the column from a separatory funnel suspended above it, at such a rate that the upper surface of the column had a continuous flow of material presented to it. It was found in initial experiments that 35 gm. of Permutit was not sufficient to adsorb all the antidiuretic substance in 35 ml. of concentrated urine, but that 2 gm. of Permutit was necessary for each ml. of concentrated urine.

The urine was allowed to flow down the column by gravity; and when the last of the concentrate had left the upper surface of the column, distilled water was allowed to flow through the column and the eluates were collected. When some 5 to 6 eluates had been collected, 5% NaCl in 1 M acetic acid was passed through the column in a manner similar to that described for the water. Again eluates were collected, the point of separation being determined by the change in the pH of the effluent, as the NaCl-acetic acid solution passed through the column. Each eluate was tested biologically for its antidiuretic properties by injection into rats.

If sufficient Permutit was used, negligible quantities of the antidiuretic substance were found in the water eluates. When the column was eluted with 5% NaCl in 1 M acetic acid, the pH of the eluates decreased from 6.8 to 3.4. No significant antidiurectic effect was found until the pH approached 4. At this pH a high level of antidiuretic activity was demonstrable. The activity of this eluate is shown graphically in Figure 1, in which the effect of the injection of 1 ml. of the eluate on the urine output of hydrated rats (solid circles) is compared to the effect of the injection of 1 ml. of distilled water (clear circles).

In the original experiment the antidiuretic activity was confined to a single eluate. In later experiments, in which the original urine concentrates had a higher antidiuretic activity, several of the eluates were found to have a pronounced antidiuretic effect. The antidiuretic activity in the later experiments was present in eluates in which the pH ranged from 5.1–3.9.

The concentrated material prepared by dialysis, insipation, and elution from a Permutit column retained its antidiuretic activity for 4 months when stood in the icebox under toluene. On standing a precipitate formed which had no antidiuretic effect. The supernatant containing the antidiuretic material was clear and dark brown in color.

Relationship of Dosage to Response of the Antidiuretic Substance. The methods of assay for the antidiuretic effects of extracts of urine,

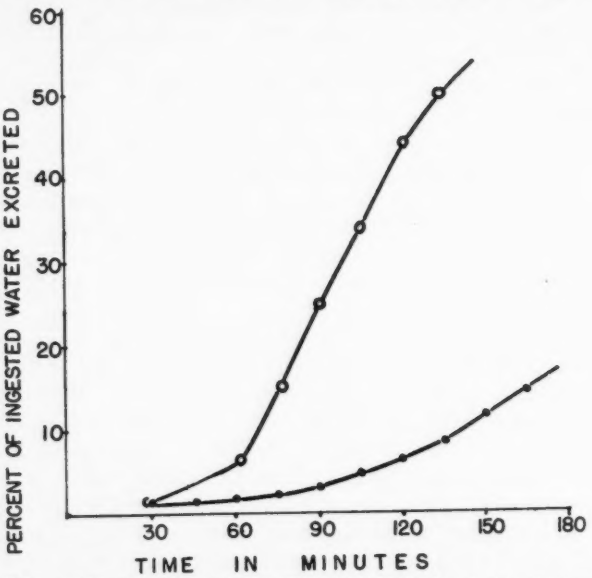

FIG. 1. The antidiuretic effect of a NaCl–CH_3COOH eluate when injected into hydrated rats as compared with the effect of 1 cc. of distilled water.

blood and tissues have depended on the suppression of urine secretion when such extracts were injected into experimental animals or man (Robinson and Farr, 1940; Teel and Reid, 1939; Ralli et al., 1945; Burn, 1931; Drill and Frame, 1948).

Jeffers et al. (1942) have shown that the response to a given dose of pituitrin is logarithmically related to the dose. The data published by Ralli et al. (1945) has been recalculated and the same relationship was found to hold true. Because of the importance of this relationship in the interpretation of the bioassays, additional experiments were carried out, the results of which are given in Figure 2. Rats that had been fasted and deprived of water for 17 hours were hydrated to 5% of their body weight by stomach tube and injected intraperitoneally with varying dilutions of commercial Pitressin. Urine was collected at 15-minute intervals for a period of 185 minutes. Over the range of

dosage studied, 2.5 to 10 milliunits per milliliter, the logarithmic relationship was found to hold, thus confirming the orginal observations of Jeffers (1942).

Effect of the Tonicity of the Solution Containing the Antidiuretic Substance upon the Response. Verney's experiments (1947) have shown that the intravenous injection of hypertonic solutions of NaCl will cause a decrease in urine flow which he attributes to an increased secretion of the antidiuretic hormone. This effect was not evoked after removal of the posterior pituitary.

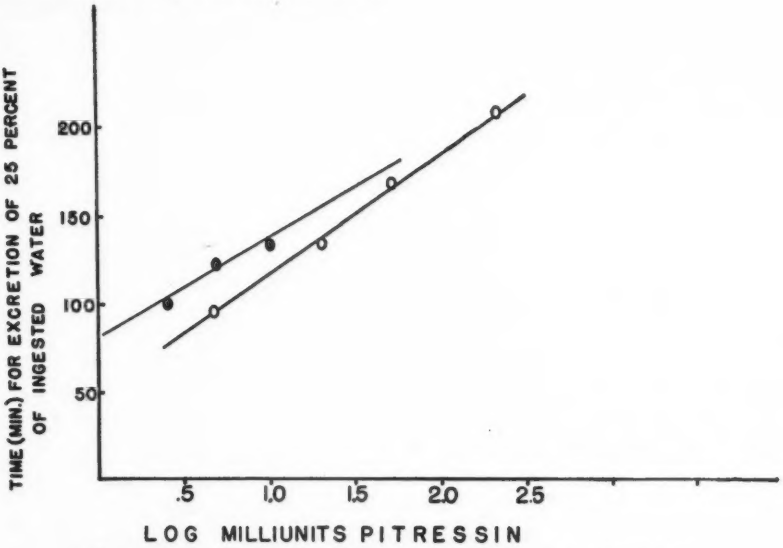

FIG. 2. Relationship of log. dosage of Pitressin to time of excretion of 25% of water ingested

(Solid circles) ..... Present Authors
(Open circles) ..... Data recalculated from Ralli et al.

We have tested the effects of the intraperitoneal injections of 1 cc. of NaCl solutions varying in concentration from 2.5 to 10%. The results are shown in Figure 3. The 5% and 10% solutions caused a decrease in urine output. The shape of the curve following the injection of the 5% NaCl solution is not characteristic of the response elicited in rats by the injection of Pitressin, although there is evidence of some antidiuretic effect. The response to the injection of 10% NaCl approximates that of Pitressin although it is not identical to it. The animals which had been given 2.5% NaCl intraperitoneally, a concentration roughly equivalent to that of the NaCl eluates from the Permutit column, excreted 25% of the injested water within a normal time.

Effect of Dialysis on Antidiuretic Activity. A solution containing the

antidiuretic substance as eluted from a Permutit column with NaCl was adjusted to a pH of 3.5 with acetic acid. (The antidiuretic hormone has been reported to be most stable at pH 3 by Van Dyke, 1943.) The solution of the antidiuretic substance was then dialyzed for 4 hours against running water, after which the material remaining in the casing was tested for its antidiuretic effect. In Figure 4 the

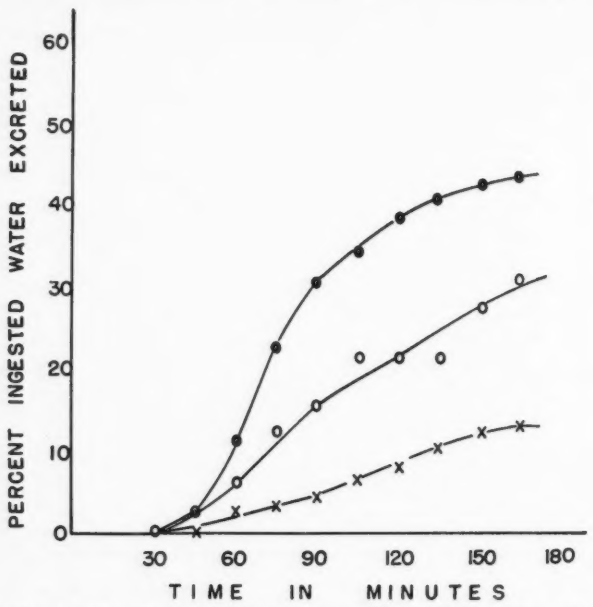

FIG. 3. Showing the effect of injection of 1 cc. of hypertonic sodium chloride intraperitoneally on the urine excretion of hydrated rats.
(Solid circles) ..... 2.5% NaCl
(Open circles) ..... 5.0% NaCl
(Crosses) ..... 10.0% NaCl

response of rats injected with a suitable concentration of the extract before dialysis is compared with that of rats injected with the same dilution of the dialyzed extract. It is apparent that a loss or destruction of antidiuretic activity occurred as a result of dialysis.

Table 3 shows the concentration of antidiuretic substance in 3 solutions before and after dialysis. The loss ranged from 50% in concentrate A, which originally contained 5 units of antidiuretic substance per cc., to 90% in concentrate C, which originally contained 40 units per cc. It is of interest that in these experiments the per cent of the antidiuretic activity lost was directly proportional to the logarithm of the concentration originally present. This relationship cannot be readily interpreted on the assumption that the loss was due to

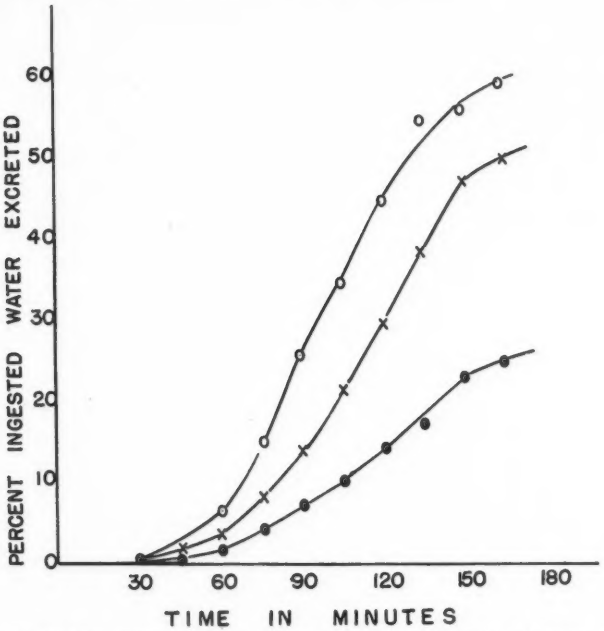

FIG. 4. Showing response of test animals to the antidiuretic substance from cirrhotic urine before and after dialysis
(Open circles) Controls injected with distilled water
(Crosses) A urine concentrate eluted from Permutit and then dialyzed
(Solid circles) The same concentrate as above, undialyzed

diffusion through the dialyzing membrane, and at present we are unable to explain it.

TABLE 3. LOSS OF ANTIDIURETIC ACTIVITY BY DIALYSIS

Urine Concentrate	Concentration of Antidiuretic Substance (Units/Ml.)		Per Cent Loss
	Before Dialysis	After Dialysis	
A	5.0	2.5	50%
B	13.9	3.9	70%
C	40.0	4.0	90%

The concentration of the antidiuretic substance is expressed as the amount equivalent to 1 milliunit of Pitressin per milliliter (Parke-Davis and Company).

DISCUSSION

From these preliminary studies we are not yet in a position to state the nature of the antidiuretic substance present in urine. The observations point to the fact that such a substance is present in urine, although with the ordinary methods of dialysis considerable portions of the substance will be lost. However, if large volumes of

urine are sufficiently concentrated, a substance can be obtained by the methods described which has a very definite antidiuretic effect.

Recently Mazur and Shorr (1948) have identified ferritin as a vasodepressor material with an antidiuretic effect. It seems unlikely that the antidiuretic activity of the extracts we have studied can be attributed to ferritin. First, except in severe kidney damage very little ferritin is excreted in the urine. Second, the antidiuretic substance which we have concentrated loses its antidiuretic effect in neutral or alkaline solutions, while ferritin is stable and exerts its antidiuretic effect at a pH of 7.4.

SUMMARY

Urines from patients with cirrhosis of the liver were collected, dyalyzed, and concentrated. The concentrates were adsorbed on a Permutit column and fractionally eluted with distilled water and with 5% NaCl in 1 M acetic acid. An active antidiuretic substance of high potency was obtained in the NaCl eluates. Dialysis reduced the antidiuretic potency of the eluates to less than half their original value. The eluates retained their potency when refrigerated for 4 months.

Although these studies do not define the nature of the antidiuretic substance present in urine, they do establish the fact that such a substance is present, that its antidiuretic effect when injected into rats is similar to that of commercial Pitressin, and that ordinary methods of dialysis may result in the loss of varying amounts of the material.

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THE EFFECTS OF PITUITARY GROWTH HORMONE ON THE METABOLISM OF ADMINISTERED AMINO ACIDS IN NEPHRECTOMIZED RATS[1]

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THE anterior pituitary growth hormone is known to promote the retention of nitrogen in the body. This effect is best seen when observations are made in animals fed adequate protein over a day or more. Anterior pituitary extracts will lower the plasma amino nitrogen of fasted animals within a few hours, an effect presumably also the result of growth hormone action. From the conditions of these experiments, it is not possible to say whether the effects seen are due to suppression of protein catabolism or to an increase in the rate of protein anabolism. An acute effect of the growth hormone on the retention of administered nitrogen has not heretofore been demonstrated. In the present experiments, the rate of increase of urea in the blood of nephrectomized rats has been used as a measure of the rate of protein metabolism. A mixture of amino acids in the form of a casein hydrolysate was given intravenously, and observations were made of the effects of a partially purified growth hormone preparation on the subsequent urea formation. No change was seen in the basal rate of nitrogen catabolism under the conditions of these experiments, but a marked decrease was observed in the amount of urea formed after the administration of the amino acid mixture. These observations suggest that the action of the growth hormone was here not so much the suppression of protein catabolism as the promotion of protein synthesis from exogenous amino acids.

METHODS

The procedure used in these experiments was similar to that recently described by Engel, Pentz, and Engel (1948), except that observations on the blood urea were begun a few hours after removal of the kidneys, instead of the next day. The lower blood urea levels at the earlier time make it possible to measure smaller differences accurately and so to observe changes within shorter time intervals than those used by Engel et al.

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The experimental animals were male white rats of the Sprague Dawley strain, about 3 months old and weighing between 300 and 350 gm. The animals had been fed on a diet of Purina Laboratory Chow. All animals were fasted overnight before experiment. The kidneys were removed through a lumbar approach, with the animals under light pentobarbital anesthesia (3 mg. per 100 gm. sodium pentobarbital given intraperitoneally) supplemented with ether during the first stages of the operation. The animals all regained consciousness fully within an hour after operation and were then allowed to rest quietly in a warm, lighted place until the later observations were begun. About 3 to 3½ hours after the removal of the kidneys, the animals were reanesthetized lightly with pentobarbital (2 to 3 mg. per 100 gm.). A blood sample was then taken from the cut tail, and immediately afterwards, the solution to be administered, the amino acid mixture or saline, was injected through the femoral vein near the groin. No further anesthetic was administered, later blood samples being taken from the tail while the animal lay quietly, wrapped in a towel.

The mixture of amino acids given was a sterile solution of material derived from casein by enzymatic hydrolysis.[2] This was dissolved as a 10 per cent solution, partially neutralized to pH 6.6 by the addition of 0.6 ml. of 0.5 N NaOH per gm. of solids. This solution was filtered by suction, dispensed into small serum vials, and immediately sterilized by autoclaving for 20 min. at 15 lb. pressure. Once tapped, a vial of amino acid solution was not used again. The solution used in most of these experiments contained per ml., 11.1 mg. of total nitrogen by Kjeldahl analysis, 7 mg. of amino nitrogen by the β naphthoquinone method, and from the manufacturer's estimate 10 mg. of potential amino nitrogen. One ml. of the amino acid solution per 100 gm. body weight was injected slowly over a period of 1 to 2 minutes. If the solution was not injected too rapidly, the animals appeared to suffer no untoward reactions, a brief period of hyperventilation being the only disturbance noted.

In control experiments, freshly prepared 0.9 per cent sodium chloride solution was injected intravenously. One-half ml. was given per 100 gm. body weight. This volume contained about the same amount of inorganic salts as was contained in the 1 ml. of amino acid solution.

Blood samples of 0.2 to 0.4 ml. volume were taken from the tail before the administration of the amino acids or salt solution and at intervals afterwards for the estimation of urea, amino nitrogen, glucose, and lactate. The total volume of blood withdrawn was from 0.8 to 1.8 ml. or not more than 0.6 ml. per 100 gm. body weight. Whole blood was used for all analyses unless otherwise indicated. In most of the nephrectomized animals, estimations of all the constituents listed were made on the initial samples; urea nitrogen was then determined at 1, 3, and 20 hours after the injections, amino nitrogen at 15, 30, and 60 minutes, glucose at 1 and 3 hours, and lactate at 30 min., 1 hr., and 3 hours. In some experiments observations of blood urea only were made on samples taken initially and at 1 and 3 hours; no differences were seen in the rates of urea formation in animals so treated from those bled more extensively. When plasma amino nitrogen was determined, samples were taken initially and at 10, 30, and 60 minutes after the injections; other determinations were not made in this series of experiments.

[2] Amigen, prepared by Mead Johnson and Company, Evansville, Indiana. We wish to thank Dr. Warren M. Cox of that company for generously supplying us with this preparation.

The blood samples were deproteinized with tungstic acid or with copper tungstate. The plasma samples were prepared as follows: About 0.4 ml. of blood was drawn into a 1 ml. centrifuge tube containing a small amount of potassium oxalate, stirred well with a fine glass rod, and spun 5 minutes in an angle-head centrifuge. Two-tenths ml. of plasma was then withdrawn with a Levi pipette, delivered into 5 ml. of 0.01 N sulfuric acid, and mixed. To the diluted plasma 0.2 ml. of 3.0 per cent sodium tungstate was then added and mixed thoroughly. The mixture was allowed to stand until settling had begun and then centrifuged. This procedure was shown to allow quantitative recovery of added amino acids estimated by the β naphthoquinone method as used here. It has been found essential for this recovery to use less tungstate in proportion to the amount of plasma than is customarily used for whole blood, since traces of tungstate not precipitated with the proteins inhibit color development during the analysis.

Chemical analyses were made by the following methods: For urea, the xanthydrol method as described by Engel and Engel (1947) was applied to 1 ml. aliquots of the supernatants prepared from whole blood by tungstic acid precipitation, or the method of Archibald (1944) was applied to samples deproteinized with copper tungstate. For amino nitrogen, the photometric procedure of Frame, Russell, and Wilhelmi as modified by Russell (1943, 1944) was used with 1 ml. of the supernatant from whole blood (1:28 dilution) or with 2 ml. of that from plasma. For glucose, the Nelson arsenomolybdate reagent was used in conjunction with Somogyi's phosphate-buffered copper reagent on 0.5 ml. aliquots, and for lactate, the Barker-Summerson method was applied to 0.3 ml. samples of the supernatants from whole blood. All determinations were made in duplicate, or in the case of urea, in some cases in triplicate.

The anterior pituitary growth hormone preparation used in these experiments was a partially purified fraction prepared by the addition of alcohol to an alkaline extract of beef anterior lobes, the "C" fraction described by Fishman, Wilhelmi, and Russell (1947). This fraction is precipitated at pH 6.8 and 24 per cent ethyl alcohol. From electrophoretic analysis, it appeared to contain approximately 40 to 50 per cent growth hormone and smaller amounts of some other components. It was highly potent in promoting growth, inducing an average gain of 21 gm. in 10 days when given at a dose of 100 μg. per day to hypophysectomized rats weighing about 100 gm. The weights of the thymuses, expressed per 100 gm. body weight, increased 50 per cent in the 10 day test, as compared to the weights in untreated controls. When given either at the 100 μg. dose for 10 days or at a dose of 1 mg. per day for 4 days, the preparation induced no changes in the weight or appearance of the adrenals, thyroids, testes, or accessory sex organs. There was therefore probably little gonadotrophin present, but there may have been some thyrotrophic hormone; from other evidence, there probably was also some lactogenic hormone. The preparation was also tested for the presence of adrenocorticotrophic hormone by the sensitive assay method of Sayers, Sayers, and Woodbury (1948). Fifty micrograms given intravenously per 100 gm. body weight to hypophysectomized rats produced an average fall in adrenal ascorbic acid of 9 ± 4 mg. per cent. Since a significant response may be induced by 0.1 to 0.2 μg. of highly purified adrenocorticotrophin, the growth hormone preparation used here appeared to be practically free of this factor.

The hormone preparation was stored in the cold in the solid lyophilized state; for use, it was suspended in 0.9 per cent sodium chloride and dissolved by adjustment of the pH to 8 to 9. This solution was prepared freshly on the day of use. A volume of 0.3 ml., containing 1 mg. of material per 100 gm. body weight, was injected intraperitoneally 1½ to 2 hours before experiment (1½ to 2 hours after removal of the kidneys).

OBSERVATIONS

Urea formation in untreated animals: A summary of the data obtained on the basal rates of urea formation in nephrectomized rats and comparison of these rates with those obtained by Engel, Pentz and Engel in similar experiments are presented in Table 1. In confirmation of the latter authors, the basal rate of urea formation was found quite constant over periods up to 22 hours after nephrectomy. This rate was about ⅓ lower than that reported by Engel, et al. The reason

TABLE 1. BASAL RATES OF UREA FORMATION IN NEPHRECTOMIZED RATS

	Length of observation period	Time after operation	No. of observations	Increase in blood urea nitrogen	
	hrs.	hrs.		mg. per cent per hour	standard deviation
I. Combined series, Tables 2 and 6*					
A.	1	3–4	23	3.38	1.09
B.	2	4–6	48	3.53	0.83
C.	16	6–22	47	3.33	0.37
II. Data from Engel, et al.**					
D.	3	16–19	201	4.8	1.6
E.	16	0–16	113	4.8	0.8

* By analysis of variance, no significant differences were found between the several experimental groups (control, growth hormone treated, adrenal demedullated, and epinephrine treated); the data from these groups were therefore pooled completely.
The animals in group A were given saline, those in groups B and C saline or amino acids at 3 hours after nephrectomy.
** Recalculated from Table 1, et seq., of Engel, Pentz, and Engel (2).

for this difference is not apparent, but it may probably be accounted for by such variables as the age of the animals, previous diet, etc. It is evidently not related to the time after operation at which the observations were made. In the present experiments the standard deviations were consistently lower then those of Engel, et al., even where the periods of observations were shorter; from the data in Table 1, it may be calculated that one-half or less the numbers of observations would be required here to obtain the same standard errors as seen in the data of the previous authors. This difference in precision may have resulted in part from the use of more recently operated animals having lower initial levels of blood urea, thus allowing the measurement of larger relative changes.

The effects of the intravenous administration of a protein hydrolysate on the formation of blood urea are shown in Tables 2 and 6. During the first hour after the injection of the amino acids, the rate

of urea formation was increased several fold over the rates seen in animals given only a saline infusion. During the second and third hours, however, the rate returned to the base line, the value in no series differing significantly from the first hour rate in the corresponding control animals.

The amounts of urea formed per 100 gm. body weight were calculated by Engel et al. on the assumption that urea is distributed in a volume of body water equal to 63 per cent of the body weight. On this basis, in animals given the same mixture of amino acids as used here, Engel et al. observed an increase over the basal rate of urea

TABLE 2. CHANGES IN BLOOD UREA IN FASTING NEPHRECTOMIZED RATS AFTER THE ADMINISTRATION OF PROTEIN HYDROLYSATE AND OF A GROWTH HORMONE PREPARATION

	No. of observ.	Increase in blood urea N mg. per cent per hour (with standard error)	
Time after injection of hydrolysate or of saline (3–3½ hours after nephrectomy)		1st hour	2d and 3rd hours
1. Saline injection*			
A. Untreated rats	8	3.4±0.40	3.9±0.15
B. Rats given growth hormone**	6	3.2±0.51	3.0±0.35
2. Amino Acid injection***			
A. Untreated rats	10	11.4±0.37	3.3±0.27
B. Rats given growth hormone	8	7.2±0.47	3.5±0.18
Difference, treated series less untreated series	18	−4.2±0.59	+0.2±0.34
3. Increase with amino acid injection over saline control series			
A. Untreated rats	18	+8.0±0.53	−0.6±0.31
B. Rats given growth hormone	14	+4.0±0.70	−0.5±0.37

* 0.5 ml. 0.9 per cent sodium chloride was given per 100 gm. body weight intravenously over 1 minute.

** 1 mg. of partially purified growth hormone per 100 gm. body weight was given intraperitoneally 1½ to 2 hours before experiment (1½ to 2 hours after nephrectomy).

*** Casein hydrolysate (Amigen) in 10 per cent solution: 1 ml. (containing 11 mg. N) per 100 gm. body weight was given intravenously during 1 minute.

formation of 4.08 mg. of nitrogen during 3 hours after the administration of 8.4 mg. of nitrogen per 100 gm. body weight. The extra nitrogen accounted for 48.5±13.3[3] per cent of that injected. As shown in Table 3, in the present experiments where 11.1 mg. of nitrogen were injected, the increment in the first hour, similarly calculated was 5.0 mg. of nitrogen per 100 gm., or 45±2.8 per cent of that administered, a figure in excellent agreement with that of Engel et al. However, it may be noted that all of the increment in rate occurred here during the first hour after the injection, and that greater precision is possible in the comparison of treated and untreated groups when the shorter time interval is used.

The effect of growth hormone on urea formation: As shown in Table 2, in the animals given the partially purified growth hormone prepa-

[3] The standard error of this figure was calculated by the present authors.

ration used here, the rate of urea formation during the first hour following the injection of saline did not differ from the control rate. In the second and third hours, there may have been a slight fall, since the difference from the corresponding control series is significant (-0.92 ± 0.38 mg. per cent per hour). However, when the hormone-treated group is compared with any of the other groups of animals given saline (Table 6), the difference is much less evident, and analysis of variance of the four groups indicates a probability of nearly 0.2 that the differences between the group means were fortuitous. There is, moreover, no difference between the rate seen in the second and

TABLE 3. THE EFFECTS OF AN ANTERIOR PITUITARY GROWTH HORMONE PREPARATION ON THE METABOLISM OF ADMINISTERED AMINO ACIDS

These figures were calculated from the increases in blood urea seen in the first hour after the administration of amino acids or of saline (given in Table 2), on the assumption that urea is distributed in water equal to 63 per cent of the body weight. All values are given per 100 gm. body weight.

Nitrogen injected, in the form of casein hydrolysate: 11.1 mg. per 100 gm. body weight.

	Urea N formed (total	N retained (not metabolized) to urea)		Urea N formed in controls given saline	Extra urea N formed
	mg./hr	mg./hr	per cent of injected N	mg./hr	mg./hr
1. Untreated animals	7.2 ± 0.23	3.9 ± 0.23	35 ± 2	2.1 ± 0.21	5.0 ± 0.33
2. Animals given growth hormone	4.5 ± 0.30	6.6 ± 0.30	60 ± 3	2.0 ± 0.32	2.5 ± 0.54
$\frac{\text{Treated}}{\text{Untreated}} \times 100$	63 ± 5*	169 ± 12			50 ± 9

* Standard errors of ratios by the form $(s_{a/b})^2 = (a/b)^2((s_a/a)^2 + (s_b/b)^2)$

third hours in the hormone-treated group and the rates seen in the first hour in any of the animals given saline. It may be doubted then that the observed decrease was in this instance a valid one. It is of course possible that larger doses of the hormone or its administration over a longer period would have induced greater changes in the basal rate of protein catabolism.

The first hour observations in the animals given the protein hydrolysate present the chief points of interest in Table 2. Clearly, the growth hormone depressed the rate of increase in blood urea following the administrations of amino acids. The decrease is equally evident whether the absolute rates are compared or whether comparison is made between the net increases in rate of urea formation over the rate seen in the respective controls given saline.

Further calculations of the amounts of urea formed and nitrogen retained (that is, not metabolized to urea) are presented in Table 3. These values are calculated on the assumption that urea is distributed in all the body water, equal in weight to 63 per cent of the body

weight. Engel, et al., have discussed the validity of this assumption. While it may be subject to some error, there is here no reason to believe that the average amount of water in the body differed in the several series of experiments. The comparison of the amounts of urea N formed are therefore nearly as accurate as indicated by the statistical evaluation. The decrease in the amount of urea formed under the influence of the growth hormone amounted here to 37 ± 5 per cent. Given the amount of nitrogen injected, the amount of nitrogen retained was calculated; an increase of 69 ± 12 per cent was observed under the influence of the growth hormone. This figure is subject to the error of the estimation of the total urea from the average body water content: if an average value of 70 per cent body water had been used, the increase would have been of the order of 100 per cent.

When the total amounts of urea formed and of nitrogen retained are considered, the assumption is implicit that the administered amino acids enter the metabolic pool and do not differ remarkably in their fate from the body amino acids ordinarily being released. Alternatively, it may be considered that the metabolism of the added amino acids is superimposed on a steady basal rate of protein catabolism. In this case, calculation of the extra urea nitrogen formed is of interest. As shown in Table 3, if this is done, the effect of the growth hormone appears to have been to decrease the rate of catabollsm of the administered amino acids by 50 ± 9 per cent.

The effects of growth hormone on the removal of amino nitrogen from the blood: The effect of growth hormone in depressing the rate of urea formation from administered protein hydrolysate could conceivably have been due to a delay in the removal of the amino acids from the blood. In order to obtain information on this point, estimations of the concentration of amino nitrogen in the blood were made at intervals following the injection of the hydrolysate or of saline. In the latter case there was observed a slight fall in the blood amino nitrogen, which may have resulted from hemodilution. In the animals given amino acids, the mean amino nitrogen level in whole blood, about 11 mg. per cent initially, was found to be near 18 mg. per cent at 15 min. after the injection, 15 mg. per cent at 30 min., and 12 mg. per cent at 60 min. Because of the rapid removal of amino nitrogen from the blood after the injection of the hydrolysate and the relatively small changes to be seen in whole blood amino nitrogen in any case, there were considerable variations in the levels of blood amino nitrogen at any given time. However, the differences from the initial levels at 30 minutes averaged about 20 per cent less in the treated group, the difference between the groups having a probability of a little more than 0.05 of being fortuitous. Evidently, the growth hormone did not delay the removal of amino nitrogen from the blood. Since the figures were in fact suggestive of some enhancement of the uptake of amino acids from the blood, another series of experiments were performed

under conditions which might be expected to make such an effect more readily observable. Intact animals were given the same growth hormone preparation, and the amino nitrogen content of the plasma followed after the intravenous administration of the protein hydrolysate. The results are given in Table 4. In the group of untreated rats were included a few nephrectomized animals, which did not differ from the intact animals in respect to the changes in plasma amino nitrogen. In these series, the increase in plasma amino nitrogen over the initial levels observed at 30 minutes after the injection of hydrolysate was about 25 per cent less in the animals given the growth hormone than in the untreated animals, and this difference was highly significant. The rate of removal of the amino acids from the plasma appears therefore to have been increased by the growth hormone.

TABLE 4. THE EFFECTS OF A GROWTH HORMONE PREPARATION ON THE REMOVAL OF AMINO NITROGEN FROM THE PLASMA OF NORMAL FASTING RATS

1 ml. of 10 per cent casein hydrolysate per 100 gm. body weight was given intravenously immediately after the initial sample was taken.

	No. of observ.	Plasma amino nitrogen—mg. per cent Initial	Increase over initial		
Time after injection of hydrolysate or of saline (3–3½ hours after nephrectomy)		0	10 min.	30 min.	60 min.
1. Untreated rats	11	7.0±0.3	+13.8	+7.3±0.51	+2.1
2. Rats given growth hormone*	8	5.2±0.3	+13.0	+5.3±0.33	+1.7
Difference		−1.8±0.40		−2.0±0.67	
t		4.5		3.0	
p		≪ .01		< .01	

* 1 mg. per 100 gm. body weight of partially purified growth hormone was given intraperitoneally 1½ to 2 hours before the initial observations.

In Table 4, it may also be seen that the growth hormone preparation had noticeably lowered the basal level of amino nitrogen in the plasma 1 to 1½ hours following the administration of the hormone.

The effects of intravenous amino acids and of growth hormone on the blood glucose and lactate: In most of the experiments on the nephrectomized rats, observations were made also of changes in blood glucose and lactate which occurred following the administration of protein hydrolysate or of saline. This was done for several reasons. The observations would serve as some indication of the metabolic state of the recently nephrectomized animals. In the event that the initial levels were normal, as proved to be the case, changes occurring after the intravenous administration of large amounts of amino acids might serve in some degree to indicate the amount of gluconeogenesis which would occur in these circumstances. Then, anterior pituitary extracts containing growth hormone have frequently been observed to cause some diminution in the blood glucose in intact rats; this process has been thought to be the result of inhibition of gluconeogenesis from body protein, but whether it would occur when amino acids were also

given has not been determined. Finally, some information was also desired concerning the possibility that the intravenous administration of large amounts of amino acids might constitute a stimulus to the sympathetic system. A summary of the most pertinent results is presented in Table 5.

The administration of the protein hydrolysate was followed by a small elevation of the blood glucose level which persisted for at least 3 hours. The growth hormone preparation lowered the blood sugar initially (first observation $1\frac{1}{2}$ to 2 hours after its administration), and in the animals given saline further depression continued during the next hour. When the hydrolysate was given to growth hormone

TABLE 5. THE EFFECTS OF PROTEIN HYDROLYSATE AND OF A GROWTH HORMONE PREPARATION ON THE BLOOD GLUCOSE AND LACTATE IN NEPHRECTOMIZED RATS*

	Blood glucose—mg. per cent				Blood lactate mg. per cent		
	No. obs.	Initial**	1 hour	3 hours	No. obs.	Initial	1 hour
A. Untreated animals							
1. Saline injection	7	91	-2 ± 2.2	$+8 \pm 4.3$	4	8.9	9.0
2. Amino acid injection	10	88	$+17 \pm 2.1$	$+15 \pm 3.0$	10	8.8	11.3
B. Animals given growth hormone							
1. Saline injection	6	79	-13 ± 3.7	-10 ± 2.5	4	13.8	18.3
2. Amino acid injection	8	81	$+9 \pm 4.1$	-4 ± 2.7	8	10.3	14.1

* Treatment as in Table 1.

** 2 to 3 hours after nephrectomy, just before the administration of the saline or hydrolysate.

The mean initial blood glucose for the 17 untreated animals was 89.4 ± 1.8, for the 14 animals given the growth hormone preparation, 80.0 ± 1.7 (determined on tungstic acid filtrates).

treated animals, little further change occurred. Since the hypoglycemic effect of the growth hormone were observed in the absence of any well defined diminution in urea production (in the animals given saline), and was equally evident whether amino acids were given or not, it seems unlikely that it resulted from depression of gluconeogenesis from protein. Recent evidence on the effects of growth hormone in diabetic animals suggests that enhanced secretion of insulin may be the cause of this hypoglycemia.

No significant changes were observed in the blood lactate in any of the experiments. This fact, together with the small changes in blood glucose, appears to indicate that there was no anoxia, massive sympathetic stimulation, or other untoward metabolic reaction to the intravenous administration of sterile protein hydrolysate.

The effects of adrenal-demedullation and of epinephrine on urea formation: As Engel et al. have noted, when non-sterile solutions of amino acids have been given intravenously or when any degree of anoxia has occurred, protein catabolism has been found to be increased (Engle, et al., 1946, 1948). The reason for these effects is unknown. Conceivably, the noxious stimuli could activate the adrenal

cortex and so cause increased release of tissue proteins. Epinephrine has been shown to stimulate the adrenal cortex and might be expected to have a similar effect upon nitrogen metabolism if given or secreted in sufficient amounts. Since the intra-venous administration of large quantities of amino acids is not a physiological procedure, it could cause sympathetic stimulation, discharge of the adrenal medulla, and also possibly consequent activation of the cortex. To test whether adrenal medullary activities were playing a part in the responses to the experimental procedures used here, a few observations were made on animals given epinephrine and on previously adrenal demedullated rats. As shown in Table 6, no significant differences from the normal were observed, either in basal rates or in the metabolism of administered amino acids. The dose of epinephrine used here was sufficient to

TABLE 6. THE EFFECTS OF THE ADMINISTRATION OF INSULIN OR EPINEPHRINE AND OF PREVIOUS ADRENAL DEMEDULLATION ON THE INCREASE IN BLOOD UREA IN NEPHRECTOMIZED RATS.

1 ml. of 10 per cent casein hydrolysate or 0.5 ml. 0.9 per cent sodium chloride per 100 gm. body weight given intravenously.

	No. of observ.	Increase in blood urea N mg. per cent per hour	
Time after injection		1st hour	2d and 3d hours
A. Animals given insulin 0.5 units per kg. intraperitoneally, 30 to 60 minutes before saline or amino acid injection			
1. Saline injection	5	4.3±0.43	5.2±0.62
2. Amino acid injection	5	11.3±0.72	4.1±0.51
B. Animals given epinephrine, 0.05 mg. per kg. intramuscularly at time of saline or amino acid injection			
1. Saline injection	6	3.3±0.55	3.2±0.50
2. Amino acid injection	4	10.0±0.78	4.0±0.42
C. Adrenal demedullated animals (3 to 4 weeks post operative)			
1. Saline injection	3	3.9±0.18	4.0±0.44
2. Amino acid injection	3	10.0±0.22	3.9±0.58

raise the blood glucose and lactate of normal fasted rats or of nephrectomized animals well above the initial levels, an average increase of 75 mg. per cent in glucose occurring in 1 to 1½ hours and an increase of 25 mg. per cent in blood lactate in about 45 minutes. Evidently this moderately large amount of epinephrine had no effect on the rate of protein catabolism within 3 hours. Since the initial blood glucose and lactate levels offer no indication that marked adrenal activity was continuing following the recent nephrectomy, it is unlikely that the failure to find any effect of epinephrine on the rate of protein catabolism was due to exhaustion of the mechanisms involved. If the administration of epinephrine were to simulate the effects of other noxious stimuli in this respect, either very much larger doses or their action over longer time intervals would be required. The fact that adrenal demedullated rats did not differ from the normal in these experiments also indicates that secretion of epinephrine, if it occurred

in response to the intravenous administration of saline or amino acids, could not normally have been influencing the results obtained.

The effects of insulin on urea formation: Insulin has been observed to increase nitrogen retention in normal animals and to diminish the rate of nitrogen excretion in fasting diabetic animals Also when insulin is given in quantity to eviscerated animals, it will partially suppress the release of amino acids from the tissues (Mirsky, 1938; Frame and Russell, 1946). Mirsky (1939) has in fact suggested that the nitrogen-sparing effects of the anterior pituitary growth factor may be mediated by an "insulinotrophic" action. In the present observations and in other unpublished experiments, the growth hormone has been seen to lower the blood sugar levels in rats in which the pancreas is intact, but not in rats which have been rendered diabetic by alloxan. In order to determine whether additional insulin secretion could have been a factor in reducing urea formation in the present experiments, a series of observations were made in nephrectomized rats given a moderately large dose of insulin. The insulin, 0.5 units per kg., was given intraperitoneally 30 to 50 minutes before the injection of the hydrolysate or of salt solution. The average blood glucose levels seen in the 10 rats so treated were 35, 36, and 61 mg. per cent (true sugar values) respectively at 0, 1, and 3 hours after the observations were begun. As shown in Table 6, no effect of this dose of insulin was seen either on the basal rate of urea formation or on that after the administration of the amino acid mixture. Since in the animals treated with insulin, the blood glucose remained lower throughout the period of observation than it did when the growth hormone was given, (about 60 to 65 mg. per cent true sugar values), it seems unlikely that the effects of the growth hormone were mediated solely through increased insulin secretion in these experiments.

DISCUSSION

The observations presented above indicate an acute effect of the growth hormone on the metabolism of administered protein hydrolysate. The rate of breakdown of the amino acids was decreased, and since the rate of removal of the amino acids from the blood was not diminished, but probably was increased somewhat, there must have occurred an increase in the retention of the administered amino acids in the tissues. Under the same conditions, little if any effect of the growth hormone was seen on the basal rate of protein catabolism. It may be suggested therefore that the growth hormone here had its most marked effect upon the anabolic phase of protein metabolism.

As to the form in which the administered amino acids were retained in the tissues under the influence of the growth hormone, no information is available from the present experiments. Earlier reports on the effects of crude anterior pituitary extracts in fasting animals indicate that the content of free amino acids (extractable with dilute

trichloracetic acid) and of peptide nitrogen in the tissues was reduced at the same time as the blood levels of amino nitrogen and urea were diminished (Schaffer and Lee, 1935). It seems likely then that the effect of the growth hormone would be to promote the formation of intracellular protein. Whether all tissues take part in this formation of protein, or whether either the liver or peripheral tissues predominate in this process, is still unknown.

The present observations indicate that extra secretion of insulin under the influence of the growth hormone is not alone responsible for the effects of the latter substance on nitrogen metabolism. However, it is still possible that some insulin may be required for the maximal effect of the growth hormone in effecting nitrogen retention. Further investigations of the relationship of insulin to the effects of the growth hormone on nitrogen metabolism are being undertaken.

SUMMARY

The rate of increase in the blood urea of nephrectomized rats was utilized as a measure of the rate of protein catabolism under basal conditions and after the intravenous administration of amino acids in the form of a sterile solution of casein hydrolysate. When a partially purified preparation of anterior pituitary growth hormone was given to nephrectomized rats 1 to 2 hours before the periods of observation were begun, the rate of urea formation during the first hour after the administration of casein hydrolysate was reduced by approximately 40 per cent and the amount of nitrogen not catabolized to urea increased by about 70 per cent. No effect of the growth hormone on the basal rate of urea formation was seen during the same time interval in animals not given exogenous amino acids.

The rate of removal of amino acids from the plasma of rats given casein hydrolysate was somewhat increased by the growth hormone.

Neither the administration of insulin (0.5 units per kg.) or of epinephrine (0.05 mg. per kg.), nor previous adrenal-demedullation affected the rate of urea formation in the basal state or after the administration of casein hydrolysate.

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INVOLUTION OF THE ADRENAL CORTEX OF RATS TREATED WITH THIOURACIL

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THERE is considerable contradiction in the literature regarding the influence of antithyroidal drugs on the adrenal cortex. Kennedy and Purvis (1941) reported adrenal hypertrophy after feeding Brassica seed, while Richter and Clisby (1942), Williams, Bissell, Jandorf, and Peters (1944), and Gordon, Goldsmith and Charipper (1944) found no effect on adrenal size. Others have expressed the opinion that any change in the weight of the adrenal gland is concurrent with a loss in body weight and have reported that if the weight of the adrenal gland is expressed as a percentage of body weight no effect is apparent (Leblond and Hoff, 1944; Williams, Weinglass, Bissell and Peters, 1944; Leathem, 1945 and 1946; Smithcors, 1945). Finally, a third group of investigators have reported a significant involution of the adrenal glands as the result of treatment with the goitrogens (Bauman and Marine, 1945; McQuillan and Trikojus, 1946; Deane and Greep, 1947), and have found hemorrhage and necrosis in the adrenal cortex (Endicott, Kornberg and Daft, 1944; Glock, 1945; Daft, Kornberg, Ashburn, and Sebrell, 1946) and loss of cytoplasmic granules (Dalton, Morris and Dubnick, 1945; Deane and Greep, 1947).

The present study was undertaken to re-investigate the question of the action of thiouracil on the adrenal cortex and to determine whether the morphological changes induced by this drug might be correlated with changes in the physiology of the gland (Tepperman, Engle and Long, 1944). Therefore, in addition to obtaining organ weights, it was decided to test the resistance of rats to cold after treatment with thiouracil for varying lengths of time. Furthermore, both immature and mature rats were included in order to determine whether the adrenal changes were due to an inhibition of growth or represented true atrophy.

MATERIALS AND METHODS

Approximately 750 rats of the Wistar Strain, inbred at the Harvard Biological Laboratories for more than 10 years, were used in the present study. The animals received a standard commercial diet and were kept at a constant temperature in an air-conditioned room. The rats received the thioura-

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TABLE 1. CHANGES IN THE RESISTANCE TO COLD AND THE SIZE OF THE ADRENAL GLAND OF THE FEMALE RAT WITH INCREASING AGE

No of rats	Age weeks	Body Wt.—Grams			Thyroid wt. mgm.	Adrenal wt. mgm.	Adrenal wt./body wt. ×100	Length of life in cold (hours)
		Start	End	Gain				
No cold								
22	3	36.0 ±0.4*			4.6 ±0.1	11.4 ±0.3	31.6 +0.8	
17	4	35.0 ±0.7	61.1 ±1.3	26.1 ±0.8	6.5 ±0.2	15.6 ±0.4	25.6 ±0.5	
20	5	35.9 ±0.4	86.3 ±1.4	50.4 ±1.3	8.7 ±0.3	21.7 ±0.8	25.1 ±0.8	
21	7	35.4 ±0.6	122.2 ±2.7	86.8 ±2.7	10.3 ±0.3	31.1 ±1.1	25.5 ±0.7	
20	9	34.5 ±0.7	150.7 ±3.9	116.2 ±4.3	10.7 ±0.4	38.2 ±2.3	25.1 ±1.1	
18	11	34.7 ±0.8	176.1 ±2.3	141.4 ±2.4	12.8 ±0.3	54.0 ±1.9	30.6 ±1.0	
17	13	36.0 ±0.7	193.7 ±3.3	157.7 ±3.1	12.4 ±0.5	52.8 ±1.9	27.4 ±1.0	
18	15	34.8 ±0.8	207.0 ±2.7	172.2 ±2.5	15.6 ±0.6	58.0 ±2.2	28.0 ±1.0	
(Exposed to Cold (3±1°C.)								
18	3	35.6 ±0.7			4.9 ±0.1	10.1 ±0.2	29.1 ±1.3	7.2 ±0.7
18	4	38.0 ±0.7	58.6 ±1.1	20.6 ±1.4	5.0 ±0.1	10.3 ±0.6	27.9 ±1.9	21.2 ±1.4
28	5	34.4 ±0.6	83.1 ±1.5	48.7 ±1.0	6.0 ±0.1	20.2 ±0.6	24.2 ±0.5	22.6 ±1.8
20	7	35.1 ±0.6	131.3 ±3.6	96.2 ±2.6	8.7 ±0.4	39.7 ±1.6	30.2 ±1.0	44.3 ±3.6
20	9	36.3 ±0.6	156.5 ±3.5	120.2 ±3.2	10.7 ±0.5	51.0 ±2.4	32.4 ±1.0	42.8 ±2.2
19	11	33.6 ±0.6	189.7 ±3.4	156.1 ±3.2	12.5 ±0.5	69.6 ±3.8	36.5 ±1.7	57.2 ±4.1
17	13	34.1 ±0.7	198.8 ±3.4	164.7 ±3.1	12.1 ±0.6	61.3 ±2.8	32.0 ±1.1	74.4 ±6.7
18	15	34.5 ±0.7	193.6 ±2.7	159.1 ±2.6	13.6 ±0.7	67.1 ±1.5	34.7 ±0.9	66.2 ±5.1

* Standard error—$Se = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

cil[2] in their drinking water as a 0.1% solution, which was freshly prepared several times a week.

In the cold-exposure experiments the animals were placed in individual wire cages to prevent huddling and put in a cold room which was maintained at 2 to 4°C. The rats were deprived of food and water during the time of exposure to cold and were examined hourly. Body weights were obtained prior to exposure, and at death the thyroids and adrenals were removed and weighed immediately on a torsion balance. The rats referred to as cretins were born from mothers treated with thiouracil for 2 weeks prior to mating and also throughout pregnancy (Hughes 1942). These animals were weaned at 4 weeks of age and continued on the drug for the duration of the study.

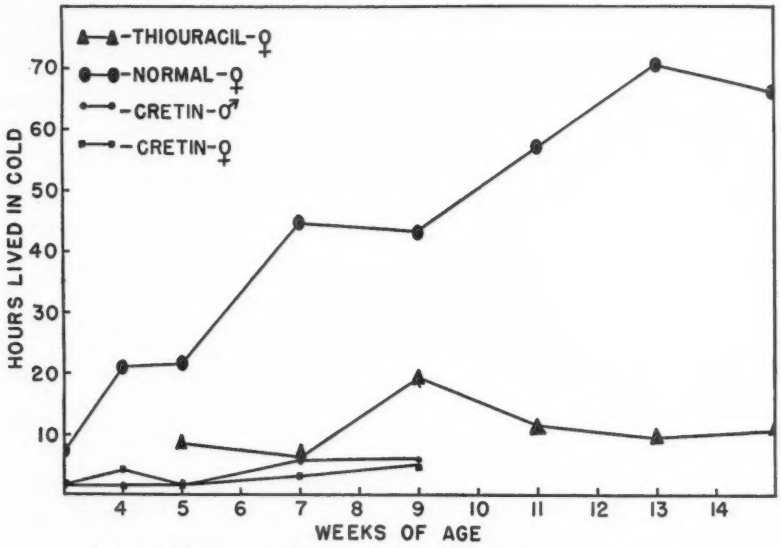

FIG. 1. Resistance of the normal, thiouracil treated, and thiouracil treated cretinoid rat to cold (2° to 4°C.) at different age levels.

Except for the experiments with adult animals and cretins, all the rats were placed on thiouracil at 3 weeks of age. The mature rats weighed approximately 200 grams (4–5 months of age) when placed on thiouracil treatment.

Statistical analyses of the data were carried out according to Snedecor (1946) for correlation coefficients and standard errors. P values were obtained from the tables of Fisher and Yates (1943).

EXPERIMENTS AND RESULTS

Normal Rats

Groups of normal female rats were exposed to a temperature of 3 ± 1°C. at 3, 4, 5, 7, 9, 11, 13 and 15 weeks of age. Similar untreated

[2] We should like to thank Dr. Stanton Hardy, Lederle Laboratories, Pearl River, New York, for the thiouracil used in this study.

control groups were sacrificed at the same time without exposure to cold. Body, adrenal and thyroid weights and length of life in the cold were obtained (Table 1). It was found that the ability of the animals to resist cold increased with age (Fig. 1). It is also interesting to note that no adrenal enlargement due to the stimulation of cold was obtained until the animals were 7 weeks of age, at which time the cold exposed rats had adrenals weighing 39.7 ± 1.6 mgms. and the controls 31.1 ± 1.1 mgms. (Table 1). This difference is also evident if the adrenal weights are expressed as a percentage of body weight. In Figures 2 and 3 it can be seen that the difference in adrenal weights of the controls and cold exposed rats, both as an absolute figure and as a percentage of body weight, is apparent at the 7th week and from that time on the adrenals of the cold exposed rats were persistently heavier. In an attempt to demonstrate a relationship between the size of the adrenal and resistance to cold, adrenal weights were plotted

TABLE 2. CORRELATION VALUES AND SIGNIFICANCE OF PROBABILITY FOR RELATIONSHIP OF LENGTH OF LIFE IN THE COLD AND ADRENAL WEIGHTS

Age weeks	Treatment	r Value	P Value
5	None	±0.663	> .001
9	None	±0.486	0.02
11	None	±0.173	<0.1
5	Thiouracil	±0.887	> .001
9	Thiouracil	±0.616	> .001
11	Thiouracil	±0.926	> .001
9	Cretin Females	±0.922	> .001

against the length of life in the cold (Fig. 4). A significant correlation with a P value of less than 0.001 (Table 2) was obtained for the group at 5 weeks of age while no significant correlation was obtained for the older groups.

Thiouracil Treated Rats

The rats used in these experiments were started on 0.1% thiouracil in the drinking water at 3 weeks of age, i.e. the time of weaning. The animals in each experiment were divided into 2 groups, of which one group was exposed to cold (3 ± 1°C.) and the other sacrificed at the same time without exposure to cold. The rats were placed in the cold room at 5, 7, 9, 11, 13 and 15 weeks of age, i.e. 2, 4, 6, 8, 10 and 12 weeks after the start of the thiouracil treatment. Body, adrenal, and thyroid weights of all animals were obtained and also the length of life in the cold for the exposed group (Table 3). The data indicate a lack of correlation between resistance to cold and age. Animals 5 weeks of age lived on the average of 8.4 ± 0.9 hours in the cold and those 15 weeks old lived 10.7 ± 1.6 hours; a difference that is definitely not significant. Comparison of the thiouracil treated animals with normal animals indicates that, while the latter show an expected

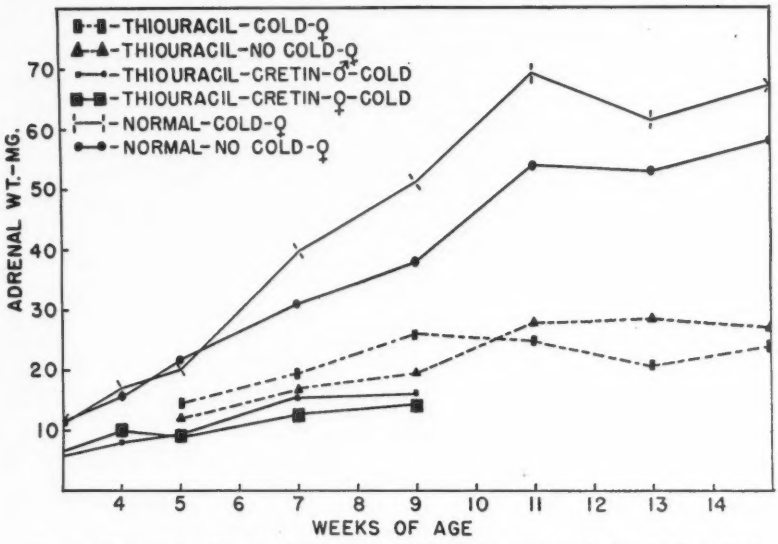

FIG. 2. Changes in the size of the adrenal gland of normal and thiouracil treated rats under normal conditions and after exposure to cold.

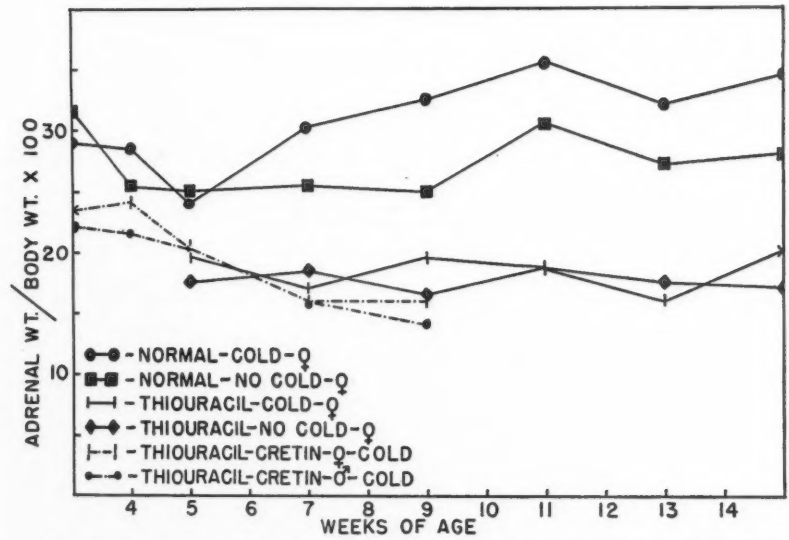

FIG. 3. Changes in the adrenal weights expressed as a percentage of the body weight in the normal and thiouracil treated rat under normal conditions and after exposure to cold.

TABLE 3. CHANGES IN THE RESISTANCE TO COLD AND GROWTH OF THE ADRENAL GLAND OF IMMATURE FEMALE RATS TREATED WITH THIOURACIL

No. of rats	Age weeks	Body Wt.—Grams			Thyroid wt. mgm.	Adrenal wt. mgm.	Adrenal wt./body wt. ×100	Length of life in cold (hours)
		Start	End	Gain				
Thiouracil for 2 weeks—No cold								
25	5	34.1 ±0.6*	70.9 ±2.1	36.8 ±1.7	29.7 ±1.1	12.4 ±0.4	17.6 ±0.4	—
Thiouracil for 4 weeks—No cold								
20	7	36.1 ±0.7	95.2 ±3.6	59.1 ±3.3	50.3 ±3.6	17.2 ±0.7	18.4 ±0.8	—
Thiouracil for 6 weeks—No cold								
19	9	35.6 ±0.7	117.5 ±3.8	81.9 ±3.7	54.6 ±2.3	19.6 ±0.8	16.7 ±0.5	—
Thiouracil for 8 weeks—No cold								
20	11	34.9 ±0.7	150.0 ±4.4	115.1 ±3.9	54.5 ±3.3	28.3 ±1.3	18.9 ±0.6	—
Thiouracil for 10 weeks—No cold								
20	13	36.7 ±0.4	165.5 ±3.4	128.8 ±3.1	58.2 ±3.6	28.7 ±1.7	17.7 ±0.8	—
Thiouracil for 12 weeks—No cold								
18	15	35.6 ±0.7	156.5 ±4.6	120.9 ±4.7	60.4 ±4.5	26.6 ±1.9	16.8 ±0.8	—
Thiouracil for 2 weeks—Cold								
40	5	36.7 ±0.4	75.9 ±1.1	39.2 ±1.3	27.6 ±3.6	14.8 ±0.1	19.4 ±0.6	8.4 ±0.9
Thiouracil for 3 weeks—Cold								
20	7	36.5 ±0.5	114.3 ±1.7	77.8 ±1.7	46.1 ±2.6	19.5 ±0.6	17.1 ±0.5	6.2 ±0.8
Thiouracil for 6 weeks—Cold								
30	9	35.7 ±0.7	130.2 ±3.8	94.6 ±3.9	47.3 ±3.9	26.0 ±1.7	19.4 ±1.0	19.0 ±3.0
Thiouracil for 8 weeks—Cold								
19	11	36.5 ±0.6	130.2 ±5.2	93.7 ±4.9	55.6 ±3.6	25.2 ±2.2	19.2 ±1.0	11.2 ±2.0
Thiouracil for 10 weeks—Cold								
19	13	36.7 ±0.6	126.9 ±7.3	90.2 ±7.5	62.4 ±2.3	21.0 ±2.2	16.0 ±0.8	9.8 ±2.0
Thiouracil for 12 weeks—Cold								
17	15	35.5 ±0.7	122.1 ±7.2	86.6 ±6.9	52.9 ±4.2	24.2 ±1.6	19.9 ±0.6	10.7 ±1.6

* Standard error.

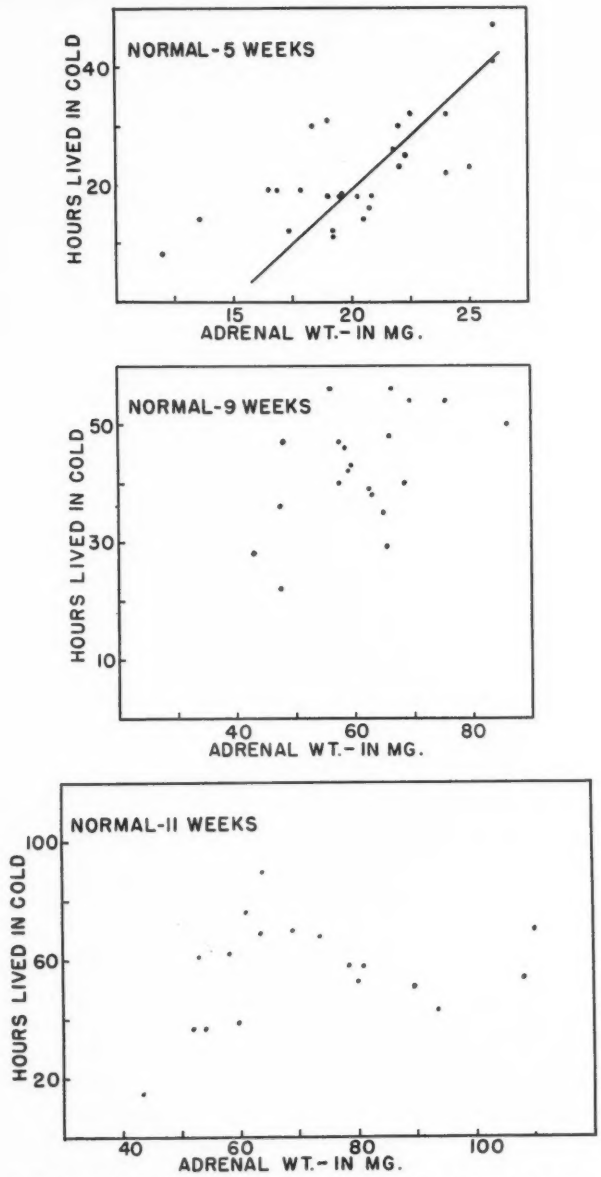

FIG. 4. Relationship between the weight of the adrenal gland of the normal female rat and its ability to withstand cold.

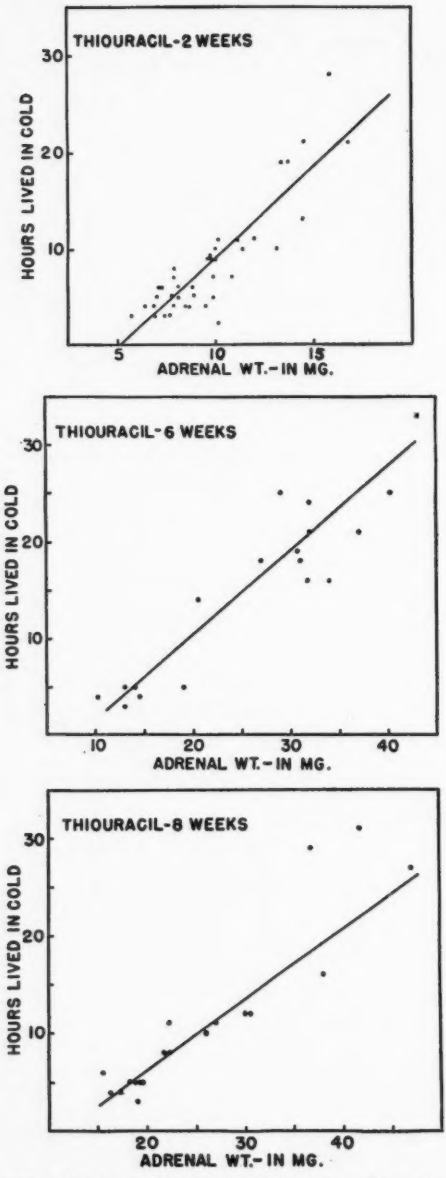

FIG. 5. Relationship between the weight of the adrenal gland of the female rat treated with thiouracil and its ability to withstand the cold.

increased ability to resist cold with increase in age, the hypothyroid animals showed no marked change in their resistance to cold (Fig. 1).

Though the weight of the adrenal gland of the thiouracil treated rats increased slightly with age, it was consistently smaller than that of the normal controls (Fig. 2). As early as the 5th week the adrenal weight of the thiouracil treated rat was 12.4 ± 0.4 mgms. whereas the controls had an average adrenal weight of 21.7 ± 0.8 (Table 3). Comparison of the adrenal weights as a percentage of the body weight

TABLE 4. THE EFFECT OF THIOURACIL TREATMENT FOR 10 WEEKS ON THE ADRENAL GLAND AND ABILITY OF THE ADULT FEMALE RATS TO WITHSTAND COLD (3 ± 1°C.)

No. of rats	Body Wt.—Grams			Thyroid wt. mgm.	Adrenal wt. mgm.	Adrenal wt./body wt. ×100	Length of life in cold (hours)
	Start	End	Gain				
Thiouracil and Cold							
13	196.0 ±2.6*	214.4 ±2.7	18.4 ±1.7	43.1 ±3.4	45.5 ±2.4	21.2 ±1.0	22.5 ±4.8
Thiouracil							
15	193.6 ±2.3	212.9 ±2.9	19.3 ±2.2	49.8 ±2.2	44.2 ±2.5	20.7 ±0.9	—
Control and Cold							
10	199.4 ±2.7	225.5 ±3.7	26.1 ±4.9	15.0 ±1.5	78.3 ±4.6	34.6 ±1.7	61.9 ±5.4
Control for End of Experiment							
13	195.2 ±3.1	230.7 ±3.7	34.5 ±2.4	18.1 ±1.2	60.5 ±2.4	26.2 ±0.8	—
Control for Start of Experiment							
13	201.2 ±2.4	—	—	15.3 ±1.1	58.4 ±1.8	28.1 ±0.8	—

* Standard Error.

showed 17.6 ± 0.4 for the thiouracil group and 25.1 ± 0.8 for the untreated controls. This inhibitory effect of thiouracil was observed in all age groups (Figs. 2 and 3). It is of further interest to note that, whereas in the normal group the adrenals of the cold exposed rats were heavier, in the thiouracil group no significant difference was noted between the cold exposed rats and their controls.

It may be seen in Figure 5 that a straight line relationship exists between the adrenal weights and the length of life in the cold. In the three groups examined (age 5, 9 and 11 weeks), a fit of data with a high significance was obtained (Table 2).

Adult Rats Treated with Thiouracil

Experiments were carried out in which adult female rats were divided into 4 groups and treated in the following manner: 1) exposure to cold after 10 weeks of thiouracil treatment, 2) controls given thiouracil for 10 weeks, 3) normal animals exposed to cold and 4) normal animals not exposed to cold.

The data indicate that the resistance to cold in the thiouracil treated animals was markedly decreased (Table 4). The normal control rats lived an average of 61.9 ± 5.4 hours while the treated rats remained alive for only 22.5 ± 4.8 hours; an unquestionably significant drop in resistance to cold. The data show also that the adrenals of the treated animals decreased in weight. This atrophy is apparent both with regard to the absolute weight of the adrenals and to the weight of the adrenals expressed as a percentage of the body weight. The

TABLE 5. FAILURE OF THE ADRENAL GLAND AND LACK OF RESISTANCE TO COLD IN THE YOUNG CRETIN RAT

No. of Rats	Age weeks	Body wt. grams	Thyroid wt. mgm.	Adrenal wt. mgm.	Adrenal wt./body wt. ×100	Length of life in the cold (hours)
			Males			
9	3	28.4 ±2.1*	12.7 ±1.73	6.2 ±0.37	22.3 ±1.23	1.3 ±0.115
14	4	38.4 ±1.54	21.6 ±1.63	8.3 ±0.397	21.6 ±0.064	1.4 ±0.0826
6	5	46.5 ±3.4	35.3 ±6.48	9.5 ±0.956	20.6 ±1.49	2.1 ±0.375
10	7	96.7 ±5.7	50.6 ±3.28	15.4 ±1.068	16.1 ±0.958	6.1 ±1.51
6	9	113.5 ±10.5	50.2 ±6.19	16.0 ±1.98	14.1 ±0.834	6.2 ±1.495
			Females			
7	3	28.4 ±2.52	14.7 ±2.17	6.6 ±0.514	23.7 ±1.51	1.4 ±0.211
23	4	37.3 ±1.86	21.7 ±1.649	10.0 ±0.71	24.3 ±1.055	3.9 ±1.137
12	5	46.8 ±3.235	25.2 ±2.92	9.2 ±0.569	20.5 ±1.369	1.9 ±0.214
9	7	80.0 ±5.3	42.4 ±4.86	12.8 ±1.23	16.2 ±1.39	3.0 ±0.593
15	9	89.5 ±8.1	48.1 ±2.84	14.6 ±1.49	16.5 ±0.84	5.4 ±1.415

* Standard error.

absolute adrenal weight in the normal rat was 60.5 ± 2.4 mgm. while the thiouracil rats had adrenals with a weight of 44.2 ± 2.5 mgm. Exposure to cold caused an increase in the adrenal weight of the normal rats (78.3 ± 4.6 mgm.) whereas no significant change was noted in the adrenals of the thiouracil treated rats (45.5 ± 2.4 mgms.). These differences are also apparent if the adrenal weights are expressed as a percentage of the body weight. As was expected the controls gained 34.7 grams in body weight whereas the treated animals gained only 19.3 grams; nevertheless, the atrophic changes in the adrenals were relatively much greater.

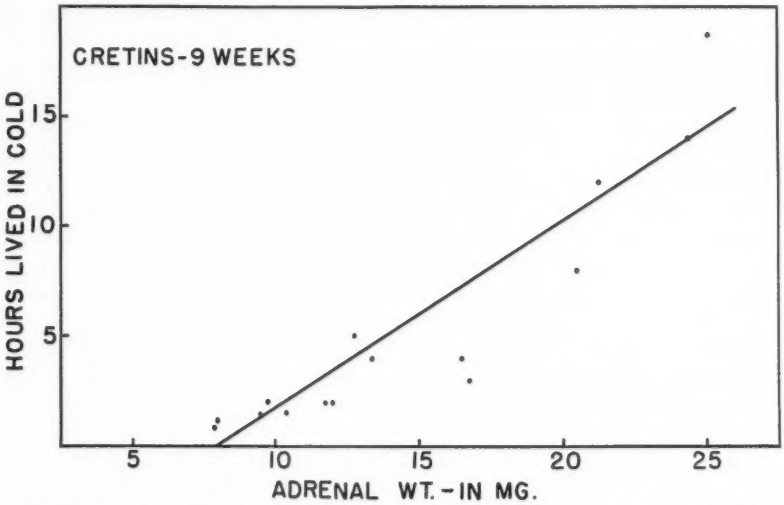

FIG. 6. Correlation between the size of the adrenal gland ability to resist cold (2° to 4°) in the thiouracil cretinoid rat. Relationship between the weight of the adrenal gland of the cretinoid rat and its ability to withstand cold.

Cretinoid Rats

Studies were carried out on a group of young cretinoid rats of both sexes. These animals were obtained from mothers that had been on thiouracil treatment for at least two weeks prior to mating and kept on thiouracil until the young were weaned. After weaning, the young were continued on thiouracil for the duration of the experiment. All litters were kept with the mother for 4 weeks in place of the usual 3 weeks in an attempt to keep the size of the young rats constant. However, in spite of this precaution large variations in weight occurred.

These animals (Table 5) showed no increase in their ability to resist cold as they grew older. They behaved in a manner similar to that found for rats started on thiouracil at the time of weaning both

with regard to their resistance to the cold (Fig. 1) and size of their adrenals (Fig. 2). The weights of the adrenals of cretinoid rats that had been exposed to cold were significantly less than those for the normal controls. As noted with the other thiouracil treated rats the cretinoids also showed a definite relationship between length of life in the cold and adrenal weights (Fig. 6) and a significant correlation for the fit of the data (Table 2).

DISCUSSION

It is apparent from the present data that definite morphological changes take place in the adrenal cortex of rats placed on thiouracil treatment. Although a contradictory literature exists with regard to this point, as was indicated in the Introduction, part of the contradiction may be due to the length of treatment. Our results indicate that it is necessary to keep the rats on thiouracil for more than 2 weeks before definite atrophic changes may be observed in the adrenal cortex. It has also been argued that any changes in the adrenal cortex are merely a reflection of loss in body weight (Leblond and Hoff, 1944; Leathem, 1945). In the present investigation though a loss in weight was encountered, nevertheless, the atrophic changes in the adrenal gland were far greater. Thus, when the adrenal weights were expressed as a percentage of the body weight, a highly significant decrease in the weight of the adrenal cortex was still found.

The loss in the weight of the adrenal cortex was obtained following treatment with thiouracil when: 1) treatment was started in the mother prior to pregnancy and continued in the young, 2) treatment was started at 3 weeks of age and 3) treatment was started after the rats had reached maturity. We may thus conclude that thiouracil not only inhibits the growth but also produces a true atrophy of the adrenal cortex.

These results are not too surprising in view of the early work of Hoskins (1910) and confirmed by Gardner (1942) that thyroid feeding causes adrenal hypertrophy and the work of Zeckwer (1938) indicating that thyroidectomy caused a loss in the weight of the adrenal cortex of the female rat. If one considers thiouracil treatment in the simplest concept as a chemical thyroidectomy one might expect adrenal involution.

The complementary action of the adrenal cortex and the thyroid in protection against cold has been apparent for some time. Such a relationship has been shown for other activities also. In 1934 Koelsche suggested a thyroid-adrenal cortex interrelationship with respect to nitrogen balance. This has been confirmed by Wells and Chapman (1940) while White and Dougherty (1947) suggest that adrenal cortical secretion has an augmenting influence on thyroid activity. Thus it would appear that a complementary thyroid-adrenal relationship

exists with regard to several activities and that with respect to cold protection antithyroidal drugs inhibit both the thyroid and the adrenal cortex.

It has been shown by numerous investigators that the adrenal cortex will protect animals against cold exposure. This procedure has been used for the assay of cortin (Selye and Schenker, 1938), DCA (Zarrow, 1942) and 11-oxycorticosteroids (Dorfman, Shipley, Schiller, and Horwitt, 1946). The latter have also shown that the 11-oxycorticosteroids are far more active than the DCA compound in protection against cold. Furthermore, Deane and Greep (1947) have indicated that in hypothyroidism, due either to thyroidectomy or thiouracil treatment, the steroid content of the adrenal as shown by histochemical techniques decreases markedly. The present results indicate that the morphological involution of the adrenal is accompanied by a physiological involution as shown by the marked decrease in the ability of the rat to withstand cold after thiouracil treatment. Ershoff (1948) has also indicated a decreased resistance in the rat after treatment with antithyroidal drugs. In view of the work of Deane and Greep (1947) it would appear that thiouracil treatment disturbs the formation of the 11-oxycorticosteroids.

It is of further interest to note that a definite relationship was obtained between the weight of the adrenal glands of thiouracil treated rats and their resistance to cold. In the normal group this relationship was found only in the 5 week old rats and not in the older age groups. This may indicate that when the animal gets older its reserve of adrenal tissue is much greater than its immediate needs, and consequently a relationship between adrenal size and function is apparent only in the normal young rat or in the thiouracil treated rat. In the latter instance, the resulting involution has probably been sufficient to diminish the reserve and thus bring out the relationship.

Failure to obtain adrenal hypertrophy in the thiouracil treated rats cannot be regarded as due to an insufficient length of exposure. Adult rats treated with the drug lived in the cold for a period of 22.5 ± 4.8 hours and it has been shown that 12 hours of exposure to stress is sufficient to cause adrenal hypertrophy (Ingle, 1938). It would appear then that this lack of response might be due either to a lack of ACTH from the pituitary or an inability of the adrenal to react to the available ACTH.

SUMMARY AND CONCLUSIONS

Treatment of young female rats with thiouracil for 6 weeks or longer results in an atrophy of the adrenal cortex. This change is not due to a loss in body weight of the treated animal for the adrenals are much lighter when expressed either as an absolute figure or as a percentage of the body weight. The same situation occurs also in the

cretinoid rat and in mature animals. Thus one may conclude that the drug not only inhibits the growth of the adrenals but also causes a true involution.

Exposure of thiouracil treated rats to a temperature of $3 \pm 1°C.$ indicates an unquestionable decrease in the ability of the animal to withstand cold. Thus the involution of the adrenal cortex after thiouracil treatment is both of a morphological and physiological nature. Within single age groups of thiouracil treated rats, a definite and constant relationship is noted between the size of the adrenal cortex and the resistance to cold. This relationship is found to exist only in the 5 week old group among normal rats. No adrenal hypertrophy occurs in the thiouracil treated rats after exposure to cold.

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INFLUENCE OF STEROID HORMONES ON GRANULATION TISSUE

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INTRODUCTION

Studies concerning the influence of steroid hormones upon connective tissue have, for the most part, been restricted to pelvic or sex organs (Loeb, Suntzeff and Burns, 1939; Gardner and Pfeiffer, 1943; Hall, 1947; Hall and Newton, 1947; Talmage, 1947; Emery and Lawton, 1947). More recently the influence of steroids, particularly desoxycorticosterone acetate, upon the body as a whole with the production of specific or systemic effects has been stressed by Selye (1943, 1944, 1946; Selye, Sylvester, Hall and Leblond, 1944). We desired to study the effects of different steroid hormones on connective tissue in laboratory animals under controlled conditions.

We became interested in one particular phase of this problem, i.e. the response of connective tissue to inflammation under the influence of selected steroid hormones. It was thought that one method would be to observe actively growing granulation tissue in experimental animals. Sterile turpentine abscesses were produced in adult and growing rats and the resultant granulation tissue in their walls studied. A comparison was then made between the response of the granulation tissue in normal animals and those which were under the influence of desoxycorticosterone acetate, testosterone propionate, or estradiol dipropionate in excess. Another series of animals were rendered comparatively free of circulating steroid hormones by the extirpation of the adrenals and gonads, and their responses were observed.

METHODS[2]

Two series of rats of the Sprague-Dawley strain were employed. The first consisted of male adults each weighing 200 grams and the second of young males and females 40 to 50 grams in weight. Nine adults were employed in each of the following groups: (1) controls, (2) those receiving 1 mgm. of

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desoxycorticosterone acetate, (3) animals receiving 2.5 mgm. of testosterone propionate, (4) animals receiving 1 mgm. of estradiol dipropionate, and (5) adrenalectomized and castrated rats. All steroid hormones were administered daily for a period of five weeks. Estradiol dipropionate and testosterone propionate were dissolved in sesame oil, desoxycorticosterone acetate in sesame or peanut oil.

Two groups, each consisting of four animals were given only peanut and sesame oil respectively, before producing abscesses.

The series of young animals, females as well as males, were divided into groups of five rats each, which were treated similarly to the above, excepting that the dosage of desoxycorticosterone acetate was 0.5 mgm., of testosterone propionate 1 mgm., and of estradiol dipropionate 0.5 mgm. Hormones were administered four times a week. Adrenalectomized-castrated animals were not employed in this series.

Using 0.5 cc. of turpentine U.S.P., abscesses were produced in all animals subcutaneously over the right scapular region. These were induced after the hormones or the oil had been administered for five weeks, and in the adrenalectomized-castrated animals three days post-operatively. One or several animals of each group were sacrificed daily beginning on the day of abscess induction.

Immediately upon sacrificing each animal the abscess was excised and fixed in a 4% formaldehyde solution. Histologic preparations were stained with iron hematoxylin and eosin, Masson's trichrome stain for the study of collagen, and Schiff's reagent after treatment of the tissue with periodic acid, for the differentiation of ground substance and "glycogen" containing elements.

The abscess walls were examined in each instance with particular reference to certain features. In order to establish a standard with which to compare the amount of fibroblastic response, a histologic constant was sought. The wall of leukocytes immediately adjacent to the turpentine was of similar thickness in all animals examined. This was designated as "L" and given a figure of one. The thickness of the fibroblastic wall was designated as "F" and used as a numerator in the ratio F/L. In addition, in an attempt to judge the exuberance of the fibroblastic response it was graded from one to five. The appearance of the fibroblasts in the controls on the fourth day of

[3] The diets consisted of Purina Dog Chow Pellets. In addition, adrenalectomized-castrated animals received 0.9% saline solution in place of tap water.

FIGS. 1–5 inclusive, ×160, illustrating granulation tissue in walls of four day old turpentine abscesses. Delimiting zone of necrosis and polymorphonuclear leukocytes on extreme left of each figure. Figs. 1a–5a inclusive, ×640, illustrating types of fibroblasts. All sections stained with iron hematoxylin and eosin.

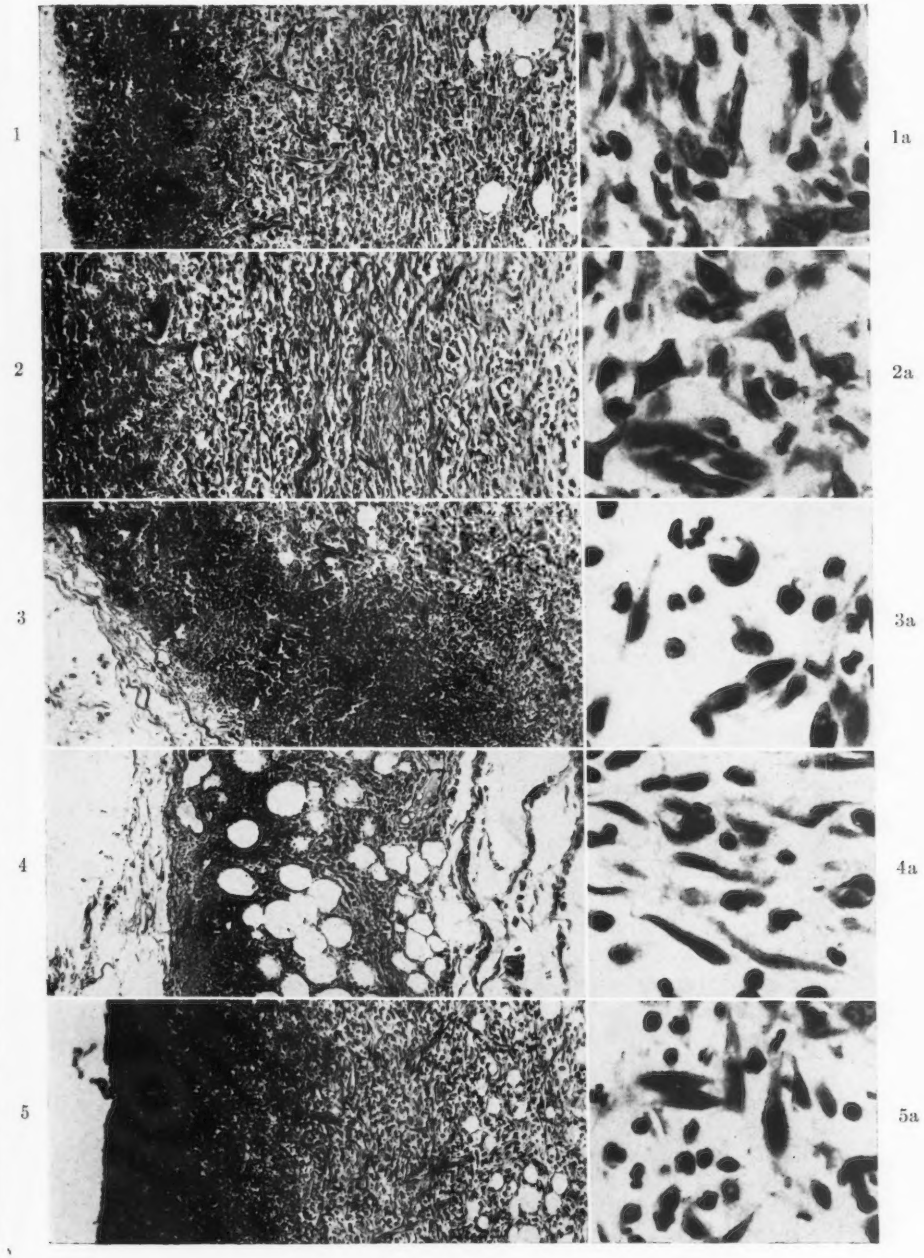
FIGS. 1 and 1a. Abscess in control animal.

FIGS. 2 and 2a. Abscess in animal receiving excesses of desoxycorticosterone acetate. Note thickness of wall of granulation tissue and large multipolar plump fibroblasts.

FIGS. 3 and 3a. Abscess in animal receiving testosterone propionate. Note thinness of fibroblastic wall and sparse, small fibroblasts.

FIGS. 4 and 4a. Abscess in animal receiving estradiol dipropionate. Note almost complete absence of granulation tissue and very thin fibrillar fibroblasts.

FIGS. 5 and 5a. Abscess in adrenalectomized-castrated animal. Note close similarity to that of control.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the turpentine abscesses was designated as three. A fibroblastic response of one and two was considered inferior and that of four and five superior to that of the controls. The types and histologic appearance of the fibroblasts were studied. In addition, the ground substance or collagen was noted from the point of view of its type of distribution and staining properties with Masson's trichrome stain and the periodic acid routine. Other factors such as the vascular response and localization of the abscesses were also noted.

RESULTS

The abscesses, even *grossly*, showed differences. Those animals in the first series which received desoxycorticosterone acetate had thicker abscess walls which were more sharply demarcated than any

TABLE 1. ADULT RATS—SUMMARY OF FOURTH AND FIFTH DAYS AFTER INDUCTION OF TURPENTINE ABSCESSES

	F/L	Fibroblastic response	Types of fibroblasts	Collagen	Miscellaneous
Controls	2	3	Large, with 2 or more processes	Moderate amount in coarse clumps	
Desoxycorticosterone acetate	4–4.5	5	Luxuriant, large, much activity, prominent nucleoli	Diffuse, homogeneous, pale staining	Granulation tissue thick, well demarcated
Testosterone propionate	1–1.5	1	Thin, spindle-shaped, sparse	Very scant. Occasional coarse clumps	Poorly demarcated abscesses
Estradiol dipropionate	0–0.5	0–1	Difficult to find. Thin, flat, small	Usually absent. Occasional delicate strands	Granulation tissue very difficult to find
Adrenalectomized-castrated	2–2.5	1–2	Thinner, smaller nuclei, less cytoplasm	Sparse coarse clumps	

of the others. In addition, the abscesses in these animals healed faster than in any of the other groups and terminated as zones of fibrosis early. The contrast was greatest grossly as well as microscopically between the desoxycorticosterone group and the rats which had received the sex hormones. The latter animals, particularly those receiving estradiol dipropionate, had abscesses which were so poorly defined that within the first four to five days they could be identified grossly only with greatest difficulty. At the termination of the experimental period, their walls were barely discernible, thin, and very poorly demarcated from the surrounding edematous subcutaneous tissue.

A summary of the microscopic picture of the abscesses in the adult rats is given in Table 1.

The *control group* of turpentine abscesses in both series went through a similar development. During the first two days there was extensive edema, moderate numbers of polymorphonuclear leukocytes and large macrophages filled with foamy material around the central accumulations of turpentine. Granulation tissue, as evidenced by thin-walled capillaries and proliferating fibroblasts was noted as early as the second day. By the third day the F/L ratio was 0.5. There were many large macrophages and the fibroblastic response was graded as

zero to one. Collagen was apparent as concentric coarse blue-staining clumps with Masson's trichrome stain. On the fourth day the F/L ratio was two. The fibroblastic response was graded as three. The cellular elements consisted of a mixture of moderately large and active fibroblasts, many thin-walled capillaries perpendicular to the center of the abscess, and macrophages. Collagen became more abundant, appeared in concentric lamellae, and was often seen in coarse clumps. There followed a gradual increase in the amount of collagen and fibroblasts. The latter increased in number but decreased in thickness and size. They assumed a more mature appearance, became elongated, thin and flattened. These alterations corresponded with an increase in collagen and the appearance of densely staining collagen in thick coarse lamellae. Inflammatory cells, except for the central zone of polymorphonuclear leukocytes and macrophages, disappeared. From the sixth day onward the F/L ratio averaged five. The periodic acid routine with Schiff's reagent added little to the observations. Pink-staining fibers and strands between fibroblasts corresponded roughly with the material staining blue with Masson's trichrome stain. The intercellular pink-staining material apparently was either part of or coincident with the collagen fibers. The only feature not revealed previously by other staining methods was the appearance of pink-staining granules within fibroblasts beginning at about the fifth day in the control group.

The animals receiving only sesame or peanut oil developed turpentine abscesses similar to the above control group.

The animals receiving *desoxycorticosterone acetate* revealed several prominent differences from those of the controls. The most pronounced was the thickness of the granulation tissue. The F/L ratio was four to four and one-half from the fourth to sixth days. The fibroblastic response was extreme. This was graded as four to five. The individual cells were large with very large nucleoli and abundant cytoplasm. Frequent mitoses were seen. These cells had numerous cytoplasmic processes which often gave them a stellate appearance. There was little or no orderly arrangement of the fibroblasts, whereas in the control group they tended to a lamellar arrangement about the central abscess cavity. The appearance of the collagen laid down between these fibroblasts was striking. It distinguished the abscesses of the desoxycorticosterone acetate animals from any of the others. The collagen was very abundant and diffuse. Instead of being arranged in clumps, it had a homogeneous, almost glassy, appearance and stained light blue with Masson's trichrome stain. The delimitation of these abscesses occurred early in their development and was very distinct. Almost complete cicatrization appeared one to two days earlier than in the controls. Capillarization was abundant and profuse.

The following differences appeared in the animals which had received *testosterone propionate* and *estradiol dipropionate*. The abscesses

in both of these were very poorly defined, their walls thin, almost completely void of granulation tissue and consisted almost entirely of polymorphonuclear leukocytes. The F/L ratio in the animals treated with testosterone propionate on the fourth day was one to one and one-half. The fibroblastic response was graded as one; the fibroblasts were sparse, very thin and spindle shaped. Collagen appeared in extremely rare, coarse clumps. The lack of response was more pronounced in rats receiving estradiol dipropionate. The F/L ratio in the first series of animals was zero to one-half. Granulation tissue was often impossible to find and fibroblasts were extremely scarce. When found they were small. Intercellular collagen was very sparse and when encountered was in thin delicate strands.

The first series also contained a group of animals which were castrated and adrenalectomized and thus were deprived of the two chief

TABLE 2. YOUNG GROWING RATS—SUMMARY OF FOURTH AND FIFTH DAYS AFTER INDUCTION OF TURPENTINE ABSCESSES

	F/L	Fibroblastic response	Types of fibroblasts	Collagen
Controls —Males	3.5	3	Moderately thick and large, moderately active	Coarse clumps, moderate amount
—Females	2	3	Moderately thick and large, moderately active but smaller than desoxycorticosterone acetate animals	Occasional fibrillar strands
Desoxycorticosterone acetate —Females	2.5	4	Large, luxuriant, active, numerous	Diffuse, pale staining
Testosterone propionate—Males	1–1.5	2	Thin, very sparse	Sparse, in clumps. In some cannot be found
—Females	2	1	Thin, sparse	Clumps, many very coarse
Estradiol dipropionate —Males	1.5–2	1–2	Very thin, sparse	Very sparse clumps—often absent
—Females	0.5–1	0–1	Thin, sparse	Sparse, in occasional thin fibers bers and clumps

sources of steroid hormones. The F/L ratio was slightly greater than the controls and averaged two and one-half in the four and five day abscesses. The fibroblasts were predominantly of the bipolar variety, tended to be smaller and flatter than those of the controls. In addition, the fibroblastic response was much less evident and was graded between one and two. Collagen was very sparse and appeared only as rare, coarse clumps.

In the series of young animals (Table 2) the response as judged by the F/L ratio was not as pronounced and there was one discrepancy as compared with the adults. The F/L ratio of a male control was three and one-half as compared with two and one-half for that of the desoxycorticosterone acetate. Despite the relatively slight differences in the F/L ratios in the two series of animals, the fibroblastic responses, types of fibroblasts, and type and distribution of collagen were similar in both. Gross changes of some significance were seen in

the animals. The gain in weight of the male controls was much more rapid, and terminally these animals weighed 85 to 100 grams more than the females which had received desoxycorticosterone acetate.

The periodic acid and Schiff's reagent routine added little to the observations. Pink-staining intercellular material paralleled in distribution and amount the collagen fibers as noted with Masson's trichrome stain. In addition, red granules were noted within some fibroblasts in greater amounts within the desoxycorticosterone animals and were practically absent in those treated with sex hormones. Whether this intracellular material was secretion produced by large macrophages or ingested glucoportein from necortic connective tissue was not determined.

Of some interest was the variation in weights of the growing young animals as summarized in Table 3. Injections of testosterone propionate and estradiol dipropionate inhibited the growth of young animals. This was particularly true of the latter hormone.

TABLE 3

	Weight in grams at start of experiment		Weight in grams at end of experiment	
	Female	*Male*	*Female*	*Male*
Controls	40–50	40–50	150–160	200–255
Desoxycorticosterone acetate	40–50	40–50	115–150	
Testosterone propionate	40–50	40–50	116–130	135–160
Estradiol dipropionate	40–50	40–50	95–100	95–105

DISCUSSION

The characteristic structure of granulation tissue in turpentine abscesses was studied in control animals as well as those receiving excesses of various hormones. Our experiments have shown that the administration of steroid hormones, i.e. desoxycorticosterone acetate, testosterone propionate, and estradiol dipropionate, and the removal of organs producing steroid hormones, have an effect on granulation tissue.

The results were outstanding in two major groups of animals. Those receiving desoxycorticosterone acetate manifested an exuberant fibroblastic response with many giant young fibroblasts and the laying down of a diffuse homogeneous groundwork of collagen. In sharp contrast, animals receiving sex hormones showed an inhibitory influence on granulation tissue with sparse and small fibroblasts and scant collagen clumps. Although of small amount in both, the collagen in the animals receiving estradiol dipropionate was of a finer, more delicate pattern and more sparse. In those receiving testosterone propionate the collagen was in coarser clumps.

Strangely enough, in castrated-adrenalectomized animals the re-

sponse of the granulation tissue was only slightly inferior to the controls.

At the present time we are unable to apply the known physiologic functions of these hormones to our observations. However, two factors must be considered. First, a possible direct influence of the administered hormones upon the proliferating granulation tissue and, second, an indirect influence. Among the latter are alterations in other mechanisms which may influence granulation tissue. In the case of desoxycorticosterone acetate, which profoundly affects water and salt metabolism, it is possible that our results are actually due to electrolytic changes. This is suggested by Selye's observations (1943) that salt-free diets inhibit the proliferation of vascular connective tissue in the kidneys in animals under the influence of desoxycorticosterone acetate. Influx of electrolytes into healing wound territory was reported previously (Andreesen and Tammann, 1933; Tammann, Bluemel and Roese, 1933). Potassium, in particular, seemed to play a major role. The part of the suprarenal cortex in these processes is unknown. Although it has been suggested (Selye, 1947) that wound healing is retarded in adrenalectomized animals and improved by administration of adreno-cortical extracts, our experiments show that even in the absence of suprarenals, granulation tissue is able to grow well.

The influence of sex hormones upon wound healing has been reviewed by Arey (1936). Earlier experiments are inconclusive. Inhibition of the anterior pituitary by excesses of sex hormones may explain the extremely poor response of the granulation tissue in animals receiving testosterone propionate and particularly estradiol dipropionate. This mechanism is suggested in our young animals in which the marked inhibition of growth of some rats receiving the sex hormones may have secondarily altered the histologic characteristics of the granulation tissue. Stunting of growth after administration of large doses of estrogenic hormones has been reported by many observers (Gardner and Pfeiffer, 1943). Doubts were raised as to whether this occurs through inhibition of the pituitary or by direct influence upon the epiphyseal cartilage (Gaarenstrom and Levie, 1939; Griffith and Young, 1942). Work is in progress to determine the exact roles of the above mentioned factors in formation of granulation tissue.

SUMMARY AND CONCLUSIONS

Granulation tissue in the walls of turpentine abscesses in normal rats as well as those receiving excessive amounts of various steroid hormones was studied. The results were compared with animals rendered relatively void of steroids through adrenalectomy and castration.

Desoxycorticosterone acetate stimulates fibroblasts and encourages the deposition of a homogeneous diffuse groundwork of collagen.

Testosterone propionate inhibits granulation tissue with the production of scant coarse clumps of collagen.

Estradiol dipropionate inhibits fibroblastic response to an even greater degree than testosterone. The collagen fibers are more delicate.

Adrenalectomy and castration of rats results in granulation tissue only slightly inferior in the fibroblastic response, the type, and amount of collagen of the control animals.

The possible factors in the production of these results were discussed.

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THE EFFECTS OF INDUCED HYPER- AND HYPO-THYROIDISM ON THE RESPONSE TO A CONSTANT DOSE OF PREGNANT MARE'S SERUM IN IMMATURE MALE RATS AND MICE[1]

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THE influence of altered levels of thyroid activity on the response to administration of gonadotrophic or gonadal hormones has not been adequately clarified. The reports available to the authors on this subject have been summarized in Table 1. It can be seen that in most instances the response of rats to the injection of gonadotrophic or gonadal hormones was increased following thyroidectomy but was decreased following thyroid or thyroxine administration. There are several exceptions, however. Grumbrecht (1939) reported an increase rather than a decrease in ovarian response to a gonadotrophin following thyroid feeding. Insofar as the response to pregnant mare's serum in the rat is concerned, Leonard and Hansen (1936) and Bischoff and Clarke (1941) noted that thyroidectomy had no effect while Smelser and Levin (1941) observed an increase in response.

Fluhmann (1934), Leonard and Hansen (1936) and Leonard (1936) stated that gonadotrophins of pituitary and non-pituitary origin may not be affected similarly by the same thyroid alterations. This would account for their finding an augmented effectiveness of sheep pituitary gonadotrophin following thyroidectomy, in contrast to an unaltered effectiveness of human pregnancy blood extract, human pregnancy urine and pregnant mare's serum. The later reports by Bischoff and Clarke (1941), Smelser and Levin (1941) and our observations do not bear out this supposition.

It is worthy of note that all workers who have dealt with the effects of altered thyroid status on the response to administered gonadotrophins have used only the rat. Conclusions have been drawn from these studies on the rat which may not be applicable to other species. In the reports on the effects of induced hyperthyroidism by feeding

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TABLE 1. EFFECTS OF EXPERIMENTALLY INDUCED HYPERTHYROIDISM AND HYPOTHYROIDISM ON RESPONSE TO GONADOTROPHIC AND STEROID HORMONES AS REPORTED BY DIFFERENT INVESTIGATORS

Investigator	Date	Animal	Thyroid Status	Type of Hormone Injected	Was Latter More (+) or Less (−) Effective
Schockaert	1931	Immature ♂ rat	Thyroidectomized	Beef pit. extract	+
Van Horn	1933	Mature ♀ castrate rat	Fed dessicated thyroid	Estrogen	−
Fluhmann	1934	Immature ♀ rat	Thyroidectomized	Sheep pit. gonadotrophin	+
		Immature ♀ rat	Thyroidectomized	Human preg. blood extract	Neither
		Immature ♀ rat	Fed dessicated thyroid	Sheep pit. gonadotrophin or Human preg. blood extract	−
		Immature ♀ rat	Injected thyroxine	Sheep pit. gonadotrophin or Human preg. blood extract	−
Leonard and Hansen	1936	Immature ♂ rat	Thyroidectomized	Pregnant mare serum	Neither
		Immature ♂ rat	Thyroidectomized	Human preg. urine	Neither
Leonard	1936	Immature ♀ rat	Thyroidectomized	Urine of castrate women	+
		Immature ♀ rat	Thyroidectomized	Urine of man with testis tumor	+
Smelser	1939	Immature ♂ rat	Injected thyroxine	Autuitrin S	−
		Immature ♂ rat	Injected thyroxine	Sheep pit. gonadotrophin	−
		Castrate ♂ immature rat	Injected thyroxine	Testis extract	−
Grumbrecht	1939	Immature ♀ rat	Fed dessicated thyroxine	"Gonadotrophic substance"	+
Bischoff and Clarke	1941	Immature ♀ rat	Thyroidectomized	Pregnant mare serum	Neither
Smelser and Levin	1941	Immature ♀ rat	Thyroidectomized	Sheep pit. gonadotrophin	+
		Immature ♀ rat	Thyroidectomized	Pregnant mare serum	+
		Immature ♀ rat	Thyroidectomized	Post-menopausal gonadotrophin	+
Williams, Weinglass, Bissell and Peters	1944	Immature ♀ rat	Fed thiouracil	Chorionic gonadotrophin	Neither
Wright and Gustavson	1947	Castrate ♀ rat	Injected thyroxine	Estrone	−
		Castrate ♀ rat	Thyroidectomized	Estrone	+
		Castrate ♀ rat	Fed thiourea	Estrone	+
Masson	1947	Castrate mature ♂ mouse	Injected thyroxine	Testosterone	+

desiccated thyroid or thyroxine, it appears doubtful that dosage levels were always adequately considered. A high dosage of thyroid hormone may have an entirely different effect on gonadotrophic activity than a low dosage, a fact which will be brought out particularly in our mouse experiment.

The objectives of the experiments reported here were (1) further to clarify the influences of induced hypothyroidism and hyperthyroidism on the response to the administration of a gonadotrophic hormone, and (2) to determine whether conclusions previously drawn from studies in the rat are also applicable to the mouse.

PROCEDURE

A total of 110 immature male albino rats of the Michigan State College strain and 120 immature male albino mice (Rockland strain) were used for these studies. Various degrees of hyperthyroidism were induced by incorporating thyroprotein[3] in the ration (Purina Laboratory Chow) in concentrations of 0.02 to 0.64 per cent, and feeding each mixture for a ten day period. Hypothyroidism was induced by including 0.1 per cent thiouracil in the ration and feeding for four to twenty day periods. Those groups of rats and mice which were to receive thiouracil for the longer periods were started at an earlier age in order to reach approximately the same body weights at the end of the experiment as the groups treated for shorter periods. The initial weights and ages of the rats averaged 39.6 to 66.4 grams and 21 to 30 days respectively, and their final weights and age were 90.8 to 111.0 grams and about 40 days respectively. The initial weights of the mice averaged 11.5 to 14.3 grams, and their final weights averaged 17.1 to 23.4 grams.

A constant dose of pregnant mare's serum (Gonadogen)[4] one-half Cartland-Nelson unit, was always injected during the last four days of thyroprotein or thiouracil feeding. Preliminary trials with several dosages of Gonadogen indicated that one-half unit produced about a hundred per cent increase in the combined weights of the seminal vesicles and coagulating glands[5] of either rats or mice. The weight increases were calculated on a 100 gram body weight basis, and the standard deviation of the mean was determined for each group. Moore (1939), Leonard and Hansen (1936), Bishop and Leathem (1946) and others have demonstrated that testis weight in immature male rats and mice is but little affected by gonadotrophin injections, whereas a marked response occurs in accessory sex glands such as the seminal vesicles and coagulating glands.

Two control groups, one untreated and the other injected with Gonadogen only, were run with each series of thyroprotein or thiouracil treated animals. The effects of thyroprotein or thiouracil alone were also determined in both rats and mice. The experiments were conducted between April and August, 1948, during which time the animals were housed in a temperature controlled room (Mean of 74°F).

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[3] An iodinated casein product ("Protamone") containing approximately three percent thyroxine as determined by chemical assay. "Protamone" was made available through the courtesy of the Cerophyl Laboratories Inc., Kansas City, Mo.

[4] Kindly supplied by the Upjohn Company, Kalamazoo, Mich.

[5] Henceforth referred to only as seminal vesicles.

TABLE 2. EFFECTS OF THIOURACIL OR THYROPROTEIN ON COMBINED WEIGHT OF SEMINAL VESICLES AND COAGULATING GLANDS OF IMMATURE MALE RATS AND MICE

Species	No. per group	Treatment	Duration days	Initial wt., g.	Final wt., g.	Testis wt., mg.	Testis wt. 100 g. final body wt., mg.	Sem.* ves. wt. mg.	Sem. ves. wt. 100 g. final body wt., mg.
Rat	5	None	10	53.0	103.7	724.6	698.7	25.1	24.4
Rat	5	0.1 % thiouracil	10	63.6	101.4	861.0	840.8	22.8	22.4
Rat	5	0.32% thyroprotein	10	59.6	110.3	992.8	900.1	26.1	23.7
Mouse	9	None	10	12.0	19.3	105.1	547.6	27.6	142.7
Mouse	7	0.1 % thiouracil	10	12.6	20.1	110.4	549.2	29.4	146.2
Mouse	7	0.32% thyroprotein	10	11.8	22.5	124.8	554.6	34.0	151.1

* Represents combined wt. of seminal vesicles and coagulating glands.

RESULTS

The effects of ten days treatment with 0.1 per cent thiouracil or 0.32 per cent thyroprotein alone on the seminal vesicles of the immature rats and mice are shown on Table 2. It can be seen that neither of these substances had any noteworthy effect on the weight of the seminal vesicles. Apparently the secretion of pituitary gonadotrophins is but little affected by altering thyroid function during this stage of life in the animals. The short treatment as well as the dosages of the two substances administered may also account in part for the negative effects.

RAT EXPERIMENTS

The effects of thiouracil on the response to Gonadogen in the rats are tabulated in Table 3. Gonadogen alone elicited a 107 per cent increase in seminal vesicle weight. Four days of feeding with thiouracil caused a slight but insignificant increase in response to Gonadogen. Ten days of thiouracil feeding almost doubled the response of the

TABLE 3. EFFECTS OF THIOURACIL ON RESPONSE TO A CONSTANT DOSE OF GONADOGEN IN IMMATURE MALE RATS

No. rats	Treatment	Initial wt., g.	Final wt., g.	Testis wt., mg.	Testis wt. 100 g. final body wt., mg.	Sem.* ves. wt., mg.	Sem. ves. wt. 100 g. final body wt., mg.	Increase over controls 100 g. body wt., %
5	Controls	61.8	110.4	606.5	549.1	26.2	23.6± .72†	
5	Gonadogen only	54.8	110.0	950.9	856.0	55.2	49.0± 8.2	+107.6
5	0.1% thiouracil for 4 days, and Gonadogen	66.4	110.6	872.9	789.2	61.3	55.4± 8.8	+134.7
5	0.1% thiouracil for 10 days, and Gonadogen	63.8	104.2	940.4	902.5	73.8	70.9± 8.6	+200.4
4	0.1% thiouracil for 10 days, and Gonadogen	60.0	103.8	1192.8	1149.1	77.4	74.5± 6.7	+211.4
5	0.1% thiouracil for 15 days, and Gonadogen	40.0	90.8	1096.9	1208.1	95.3	104.6± 6.6	+343.2
5	0.1% thiouracil for 20 days, and Gonadogen	39.6	91.9	1177.2	1208.7	106.4	115.8±24.2	+390.7

* Represents combined wt. of seminal vesicles and coagulating glands.

† Standard error of the mean = $\sqrt{\frac{\sum d^2}{n(n-1)}}$.

TABLE 4. EFFECTS OF THYROPROTEIN ON RESPONSE TO A CONSTANT DOSE OF GONADOGEN IN IMMATURE MALE RATS

No. rats	Treatment	Initial wt., g.	Final wt., g.	Testis wt., mg.	Testis wt., 100 g. final body wt., mg.	Sem.* ves. wt., mg.	Sem. ves. wt., 100 g. final body wt., mg.		Increase over controls, 100 g. body wt., %
5	Controls	43.6	92.7	625.1	674.3	18.0	19.5±1.5†	av. = 24.3	
11	Controls	52.0	106.7	1070.8	1003.0	31.2	29.2±1.9		
5	Gonadogen only	43.2	95.1	848.0	891.3	53.0	55.7±2.5	av. = 50.2	+106.5
5	Gonadogen only	44.3	88.6	625.8	706.3	39.7	44.8±1.6		
5	0.02% thyroprotein, and Gonadogen	41.2	89.1	666.0	747.7	46.5	52.2±2.6		+114.5
5	0.04% thyroprotein, and Gonadogen	50.6	105.3	910.8	865.3	49.9	47.4±2.3		+ 94.7
5	0.08% thyroprotein, and Gonadogen	44.3	88.1	607.8	689.9	29.6	33.5±3.1		+ 46.0
5	0.16% thyroprotein, and Gonadogen	50.0	97.0	737.2	760.3	30.6	31.6±2.9		+ 29.8
5	0.32% thyroprotein, and Gonadogen	58.8	109.1	1036.0	949.6	40.8	37.3±6.6	av. = 36.0	+ 47.8
5	0.32% thyroprotein, and Gonadogen	45.6	88.8	704.5	793.1	30.9	34.8±2.5		
5	0.64% thyroprotein, and Gonadogen	51.0	106.5	821.1	770.8	30.1	28.3±2.9		+ 16.0

* Represents combined wt. of seminal vesicles and coagulating glands.
† Standard error of the mean.

seminal vesicles to Gonadogen injection. The fifteen and twenty day treatments with thiouracil at least tripled the response to Gonadogen. It is evident that as the degree of thiouracil-induced hypothyroidism was increased in the rat, the greater was the response to a constant dose of pregnant mare's serum.

Thyroprotein feeding reduced the effectiveness of Gonadogen (Table 4). Gonadogen alone elicited a 107 per cent increase in weight of the seminal vesicles. The addition of 0.02 or 0.04 per cent thyroprotein produced no significant alteration in gonadotrophic response, but higher concentrations progressively inhibited the response. The highest level of thyroprotein fed, 0.64 per cent, appears to have completely inhibited the action of the pregnant mare's serum on the seminal vesicles.

TABLE 5. EFFECTS OF THIOURACIL ON RESPONSE TO A CONSTANT DOSE OF GONADOGEN IN IMMATURE MALE MICE

No. mice	Treatment	Initial wt., g.	Final wt., g.	Testis wt., mg.	Testis wt., 100 g. final body wt., mg.	Sem.* ves. wt., mg.	Sem. ves. wt., 100 g. final mg.	Increase over controls 100 g. body wt., %
8	Controls	12.5	22.8	158.2	693.3	36.2	158.5±14.7†	
6	Gonadogen only	13.3	17.3	111.9	648.3	55.2	321.7± 4.9	+102.9
8	0.1% thiouracil for 4 days, and Gonadogen	13.7	17.1	113.4	661.3	38.2	222.9±17.4	+ 40.6
8	0.1% thiouracil for 7 days, and Gonadogen	13.2	21.1	125.0	592.8	42.7	202.7±21.5	+ 27.8
8	0.1% thiouracil for 10 days, and Gonadogen	12.2	20.4	125.7	616.3	50.7	248.4±23.2	+ 56.7
8	0.1% thiouracil for 15 days, and Gonadogen	12.0	21.8	143.0	657.0	51.4	236.1±14.2	+ 48.9
6	0.1% thiouracil for 20 days, and Gonadogen	11.5	20.9	154.7	139.6	53.6	256.5±22.3	+ 61.8

* Represents combined wt. of seminal vesicles and coagulating glands.
† Standard error of the mean.

MOUSE EXPERIMENTS

In contrast to the rat, the effectiveness of Gonadogen in the mouse was reduced by thiouracil and augmented by thyroprotein feeding. In the thiouracil series (Table 5), the pregnant mare's serum alone caused a 103 per cent increase in seminal vesicle weight. The thiouracil treated groups all showed a significantly reduced capacity to respond to Gonadogen, although in no group was the response completely inhibited.

The feeding of thyroprotein caused an increase in response to Gonadogen in all groups, with the exception of the group fed the highest concentration of thyroprotein, 0.64 per cent (Table 6). The latter level of thyroprotein slightly reduced the effectiveness of Gonadogen, emphasizing the importance of dosage levels of thyroidal substances. In general, it can be stated that immature male mice and immature male rats react oppositely to the same gonadotrophic stimulus during similar alterations in thyroid function.

TABLE 6. EFFECTS OF THYROPROTEIN ON RESPONSE TO A CONSTANT DOSE OF GONADOGEN IN IMMATURE MALE MICE

No. mice	Treatment	Initial wt., g.	Final wt., g.	Testis wt., mg.	Testis wt., 100 g. body final wt., mg.	Sem.* ves. wt., mg.	Sem. ves. wt., 100 g. final body wt., mg.	Increase over controls 100 g. body wt., %
9	Controls	12.0	19.4	105.1	547.6	27.6	142.7±10.6†	
9	Gonadogen only	11.6	20.3	116.4	572.6	46.1	226.9±19.5	+ 58.9
6	0.02% thyroprotein, and Gonadogen	12.0	22.1	133.3	602.7	76.1	344.5±12.7	+141.4
6	0.04% thyroprotein, and Gonadogen	13.6	23.3	140.7	603.7	77.8	333.9±17.1	+134.0
6	0.08% thyroprotein, and Gonadogen	14.3	23.4	129.3	551.8	74.6	318.5±31.8	+123.2
5	0.16% thyroprotein, and Gonadogen	14.0	22.1	141.1	637.9	72.6	328.0±18.9	+129.9
5	0.32% thyroprotein, and Gonadogen	14.2	22.4	145.0	647.7	64.5	288.2±24.6	+101.9
8	0.64% thyroprotein, and Gonadogen	12.7	21.2	134.0	630.9	41.6	195.9±11.7	+ 37.2

* Represents combined wt. of seminal vesicles and coagulating glands.
† Standard error of the mean.

DISCUSSION

It cannot be maintained that the normal immature male rat is "hyperthyroid" or that the normal immature male mouse is "hypothyroid," since such terms are more properly reserved for individuals with particular syndromes rather than for an entire species. The data presented here indicate that, insofar as the response to pregnant mare's serum is concerned, immature male rats are secreting more than an optimal amount of thyroid hormone, whereas immature male mice are secreting less than an optimal amount (Fig. 1). Studies of the thyroid secretion rates of rats (Monroe and Turner, 1946) and mice (Hurst and Turner, 1948) do not reveal any marked differences between the two species. However, other lines of evidence substantiate the idea that the immature rat is secreting more than an optimal amount of thyroid hormone for certain processes when compared to the immature mouse.

Astwood (1945) demonstrated that the administration of 0.01 per cent thiouracil in the feed of rats for a period of 9.5 months beginning at weaning time increased the growth rate over controls. This increase was reflected in a proportional gain in skeletal dimensions. In mice, Hurst and Turner (1948) reported that thiouracil fed at several levels invariably retarded growth, and this was reflected in a greater fat content of the carcasses. The administration of thyroxine or thyroprotein by Koger and Turner (1943) was found to retard growth in rats but to increase growth in mice. The increased body weight of the mice was reflected in a greater retention of protein and water and a reduction in fat content. A recent report by Fogelman and Ivy (1948) indicates that rats are secreting more than an optimal amount of thyroid hormone for another growth process. They noted that administration of thiouracil to partially hepatectomized rats increased the rate of liver regeneration when compared with controls.

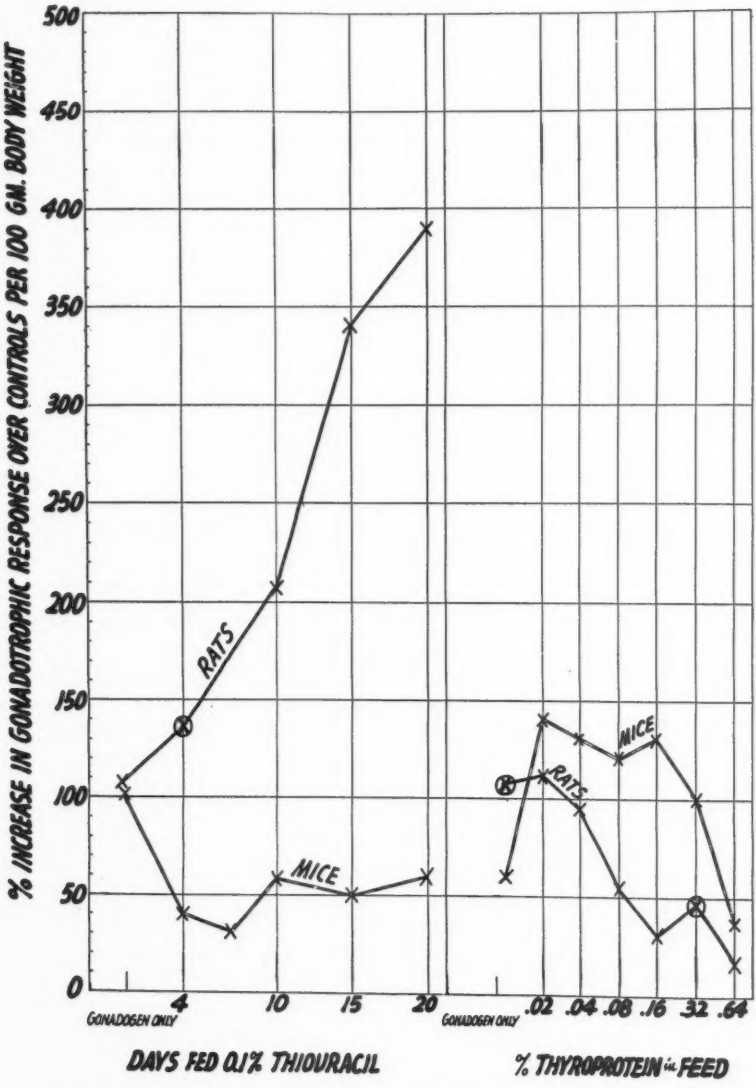

FIG. 1. The percent increase in the combined weights of the seminal vesicles and coagulating glands following treatment with Gonadogen only, and with Gonadogen plus thiouracil or thyroprotein.

⊗ Average of two groups.

Recent work on mammary growth in rats and mice further illustrates the dissimilarities in the effects of experimentally altered thyroid function in these species, Leonard and Reece (1941), Smithcors and Leonard (1943), Trentin, Hurst and Turner (1948) and Johnston and Smithcors (1948) found that thyroidectomy or thiouracil administration augmented mammary growth in the rat. However, similar treatment in the mouse was found to decrease mammary growth by Mixner and Turner (1943), Mixner (1947) and Trentin, Hurst and Turner (1948). Mixner and Turner (1943) also reported that the administration of thyroxine to ovariectomized mice increased the mammary growth response to injections of estrogen and progesterone.

All of the above findings appear to support the conclusion that growing rats secrete more than an optimal amount of thyroid hormone while growing mice secrete less than an optimal amount insofar as the reactivity of certain body tissues and organs are concerned. A different theory, however, was offered by Bischoff, Clarke and Epps (1941) to explain the effects of thyroidectomy or thyroid administration on the response to gonadotrophins in rats. They believed the altered thyroid status either decreased or increased the exchange of body fluids, thus decreasing or increasing the rate of hormone resorption. The observations in the mouse, however, appear to make such a conclusion untenable. Obviously it is illogical to suppose that thyroid administration increases the rate of exchange of body fluids in the rat but decreases it in the mouse.

It is believed the data presented in this paper, as well as related work of other investigators, definitely establishes the existence of an important species difference between young rats and mice. It can be stated that young rats secrete more than an optimal amount of thyroid hormone while young mice secrete less than an optimal amount of thyroid hormone for a variety of body functions and reactions. This suggests that other species, and perhaps some strains and individuals within a species, also may not be secreting optimal amounts of thyroid hormone for certain body functions. There is the possibility, therefore, of inducing degrees of hypothyroidism or hyperthyroidism which would insure maximum responsiveness to certain stimuli. These possibilities are already being explored in the field of animal research, where thiouracil and thyroprotein are being fed at various levels to farm animals in an attempt to increase productive processes such as growth, fattening, milk yield and egg production.

SUMMARY

The effects of experimentally induced hyper- or hypothyroidism on the response to a constant dose of pregnant mare's serum ("Gonadogen") were determined in immature male rats and mice. Hypothyroidism was produced by feeding 0.1 per cent thiouracil for four to twenty day periods and hyperthyroidism was produced by feeding

thyroprotein in concentrations of 0.02 to 0.64 per cent for ten day periods. The increase in the combined weight of the seminal vesicles and coagulating glands was used to measure the response to the pregnant mare's serum.

Thiouracil or thyroprotein, when fed alone, had no effect on the weight of the seminal vesicles and coagulating glands. When pregnant mare's serum was injected, the response of the seminal vesicles and coagulating glands was partially to completely inhibited by all except the lowest levels of thyroprotein in the rat, while in the mouse all except the highest concentration of thyroprotein increased the gonadotrophic response by 72 to 140 per cent. Thiouracil, particularly when fed for the longer periods, increased the gonadotrophic response in rats by as much as 300 per cent, while the response in mice was reduced by 40 to 73 per cent.

It is concluded that, insofar as the response to pregnant mare's serum is concerned, immature male rats are secreting more than an optimal amount of thyroid hormone whereas immature mice are secreting less than an optimal amount of thyroid hormone. A discussion of these and related data by other investigators leads to the conclusion that there is a distinct species thyroid difference between the rat and mouse which accounts for their opposite reactions to a number of similar stimuli.

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THE QUANTITATIVE RELATION BETWEEN CERTAIN AMINO ACIDS AND GLYCOGENESIS AS INFLUENCED BY ADRENALECTOMY AND ADRENAL REPLACEMENT[1]

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INTRODUCTION

THE effect of adrenal cortical function upon carbohydrate and protein metabolism has been repeatedly demonstrated. Much evidence has accumulated to indicate that there is a direct effect of certain cortical principles upon the rate of gluconeogenesis from protein, but there is little knowledge as to the reactions involved. Several possible mechanisms have been postulated. Long, Katzin and Fry (1940) suggest that the cortical principle accelerates the mobilization of endogenous proteins. Wells and Kendall (1940) have reported that the phlorhizinized, adrenalectomized rat forms glucose from fed protein at a normal rate. Further evidence in favor of this mechanism is offered by Evans (1941), who found that the rate of deamination of alanine proceeded at a normal rate in the adrenalectomized rat.

The possibility that the cortical hormones exert their effect upon the rate of deamination of amino acids was investigated by Lewis, et al (1940). They observed that the formation of glucose from alanine in the adrenalectomized, phlorhizinized rat was reduced. Russell and Wilhelmi (1941) found that kidney slices from adrenalectomized rats produce less than normal amounts of carbohydrate from alanine and glutamic acid. Jimenez—Diaz (1936) reported a subnormal rate of deamination in the kidneys of adrenalectomized cats. Samuels, et al (1937) found that cortical insufficiency in the rat caused a decrease in the formation of liver glycogen from fed alanine. These and other prevailing theories are discussed at length by Long (1941).

A new approach to this problem was offered by the development of quantitative paper chromatography (Awapara, 1949) for the estimation of some free amino acids in tissues. It appeared important to extend, by the use of this technique, the study of the role of the

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adrenal glands in the conversion of protein to glycogen. The results of a study of liver glycogen and alanine, glycine, glutamic acid and aspartic acid in hepatic and muscle tissue of normal rats, untreated adrenalectomized rats, and adrenalectomized rats given substitution therapy are herewith presented. The replacement materials consisted of 17-hydroxydehydrocorticosterone and desoxycorticosterone acetate, referred to subsequently as Compound E (Kendall) and DOCA, respectively.

EXPERIMENTAL

Male rats, of the Sprague-Dawley strain, weighing 130–145 gm., were used in this study. The adrenal glands were removed under light ether anesthesia through separate incisions. Care was exercised to remove as much of the periadrenal adipose tissue as possible, and each adrenal gland was inspected after excision for gross evidence of incomplete removal. The body wall was closed with gut, and the skin incision repaired with skin clips. Reasonable asepsis was observed during the procedure.

Immediately following the operation, the rats were given physiological saline and ordinary food *ad libitum*. The animals were weighed at the time of the operation, and twice during the subsequent six day period. Only those animals surviving and showing a weight gain during this period were used.

The adrenalectomized rats receiving either Compound E or DOCA were given distilled water for drinking purposes during the 24 hour fasting period preceding autopsy. The third group of adrenalectomized rats received no treatment other than physiological saline during the fasting period. Three mg. of Compound E and 1.0 mg. of DOCA in 1.5 ml. and 0.6 ml. of corn oil, respectively, were given each rat of the appropriate groups. The total dose was given in three, equally divided, subcutaneous injections during the first 8 hours of the 24 hour fast.

At the time of autopsy, the rats were killed with preliminary anesthesia by a lethal, intraperitoneal injection of nembutal. A sample of liver weighing approximately 1 gm. was quickly removed and placed in hot 30 per cent potassium hydroxide for hepatic glycogen determinations. A second liver sample was taken for the amino acid determinations. The remainder of the liver of each alternate rat was dried for 24 hours at 100°C. in order to determine the percentage of total solids. A sample of the gastrocnemius muscle of each rat was also removed for determination of amino acids.

Each rat was carefully examined for residual adrenal tissue macroscopically and none of the operated rats was found to have such. The rats receiving Compound E and the controls lost practically twice as much weight during the 24 hour fast, as did the rats receiving desoxycorticosterone acetate or only salt water during the same period.

Liver glycogen was determined by the method of Good, Kramer and Somogyi (1933). Amino acids were extracted from tissue by the method of Awapara (1948) and measured by the technique of quantitative paper chromotography (Awapara, 1949).

RESULTS AND DISCUSSION

In Table 1 are shown the results of analyses for glycogen in liver and for free amino acids in liver and muscle of the experimental and

normal control animals. The increase of glycogen in the livers of Compound E-treated animals over that of normal rats, adrenalectomized rats maintained on sodium chloride, or rats treated with DOCA, is confirmatory of many previous observations. There is a striking decrease in the concentration of free dicarboxylic amino acids in the liver following administration of Compound E. This is in contrast to the results obtained by desoxycorticosterone acetate or saline treatment. The diminution in concentration of these amino acids as compared

TABLE ONE

STATUS — GIVEN	LIVER GLYCOGEN PER CENT	LIVER (micrograms of amino nitrogen per gram fresh tissue)				MUSCLE (micrograms of amino nitrogen per gram fresh tissue)			
		SUM	DI-COOH ACID FRACTION	GLYCINE	ALANINE	SUM	DI-COOH ACID FRACTION	GLYCINE	ALANINE
INTACT — NONE	0.95 ±0.17*	313 ±18.13	119 ±5.10	107 ±12.80	87 ±9.01	428 ±39.99	58 ±2.70	279 ±34.17	91 ±12.11
ADRENAL — COMP. E	1.50 ±0.38	211 ±13.11	78 ±4.22	83 ±8.37	50 ±3.37	315 ±11.88	58 ±9.21	203 ±11.04	55 ±4.64
ADRENAL — D.O.C.A.	0.15 ±0.02	255 ±19.09	140 ±18.78	67 ±11.52	49 ±5.00	298 ±18.84	38 ±3.49	202 ±18.11	59 ±2.51
ADRENAL — SALINE	0.19 ±0.05	321 ±26.65	167 ±13.30	93 ±11.70	61 ±4.12	293 ±14.91	43 ±3.30	180 ±11.32	69 ±5.27

*STANDARD ERROR CORRECTED FOR SMALL NUMBERS

TABLE 1. CONCENTRATION OF GLYCOGEN AND FREE AMINO ACIDS IN LIVER AND MUSCLE

with the results in normal, unoperated animals is less marked, but roughly proportional to the glycogen differences demonstrated between these two groups. The changes in amino acid concentration and distribution in muscle were statistically insignificant. This finding corresponds to the moderate changes, and often difficultly demonstrable effects of adrenalectomy and steroid hormone treatment on muscle glycogen.

In Table 2 the amino acid concentrations are recorded with reference to the total free amino acid nitrogen represented by the summation of glutamic acid, aspartic acid, alanine and glycine. The only striking observation to be made here is the relative excess of free

alanine in the livers of unoperated controls and Compound E-treated, adrenalectomized rats.

An abundance of previous work demonstrates that adrenal cortical insufficiency is associated with impaired glycogenesis from protein. Normal conversion of protein to carbohydrate is restored in the adrenalectomized animal by administration of the C-11 oxygenated corticosteroids. The 11-desoxycorticosteroids, although highly active with respect to electrolyte and water metabolism, do not stimulate

TABLE TWO

GROUP	$\Sigma F = 1.00$	DI-COOH FRACTION	GLYCINE	ALANINE
	RATIO OF EACH FRACTION OF LIVER TO SUM OF LIVER FRACTIONS			
NORMAL	1.00 ±0.083	0.38 ±0.024	0.34 ±0.046	0.28 ±0.033
COMP. E	1.00 ±0.087	0.37 ±0.031	0.39 ±0.046	0.24 ±0.022
D.O.C.A.	1.00 ±0.106	0.55 ±0.084	0.26 ±0.049	0.19 ±0.024
SALINE	1.00 ±0.116	0.52 ±0.059	0.29 ±0.044	0.19 ±0.020
	RATIO OF EACH FRACTION OF MUSCLE TO SUM OF MUSCLE FRACTIONS			
NORMAL	1.00 ±0.127	0.14 ±0.015	0.65 ±0.099	0.21 ±0.034
COMP. E	1.00 ±0.053	0.18 ±0.029	0.64 ±0.042	0.18 ±0.017
D.O.C.A.	1.00 ±0.089	0.13 ±0.014	0.68 ±0.074	0.20 ±0.015
SALINE	1.00 ±0.072	0.15 ±0.015	0.61 ±0.050	0.24 ±0.022

S.E. OF QUOTIENT = $Q\sqrt{(S.E._1/M_1)^2 + (S.E._2/M_2)^2}$

TABLE 2. RATIOS OF INDIVIDUAL AMINO ACIDS TO TOTAL AMINO ACIDS DETERMINED

the formation of glycogen from non-carbohydrate sources. Experimental evidence presently available does not provide unequivocal support for any theory attempting to explain the mechanism, nor does it identify the amino acids through which the 11-oxycorticosteroids stimulate glycogenesis. However, the well established position of the dicarboxylic amino acids and alanine as intermediates in the transformation of protein to carbohydrate through the aminopherase (transamination) mechanism suggests a possible explanation for the data recorded in Table 1. The action of Compound E in promoting glycogen synthesis was accompanied by a diminished concentration of the free dicarboxylic amino acids of liver tissue. This finding may

reasonably be interpreted as indicating the transformation of these amino acids to carbohydrate. This conclusion is supported further by the data recorded in Table 2. Here we note that glycogen synthesis is accompanied by a relative increase in the proportion of alanine to the dicarboxylic amino acids. This finding is explicable in terms of the aminopherase mechanism, since the reaction rates of both glutamic acid and aspartic acid with pyruvic acid in liver favor the production of alanine. Our figures for amino acid distribution in muscle may be correlated with the fact that the aminopherase reactions proceed at low, inappreciable rates in that tissue.

Although these findings suggest that carbohydrate synthesis stimulated by Compound E takes place through the aminopherase mechanism and involves the utilization of the dicarboxylic amino acids, more complete studies will be necessary to establish this fact. Cohen has pointed out that in the field of intermediary metabolism, final identification of a process requires not only a determination of change in the reacting substances, but also a demonstration of the enzyme mechanism concerned.

The absorption, transport and deposition of carbohydrate in the adrenalectomized animal is known to approach the normal so long as maintenance amounts of sodium chloride are fed or there is adequate treatment with the 11-desoxycorticosteroids. If, however, animals so treated are subjected to fasting or any other conditions requiring the carbohydrate stores to be replenished from protein sources, the C-11 oxygenated corticosteroids must be available if an effective adaptation is to be accomplished. The conditions of our experiment, imposing only a 24 hour fast, are not calculated to show the maximum deficiency in carbohydrate metabolism in the animals deprived of the 11-oxysteroids. No doubt, the trends exhibited here could be considerably magnified by applying more strenuous conditions.

ACKNOWLEDGMENT

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SUMMARY

The increase in liver glycogen of Compound E-treated, adrenalectomized rats over normal rats, or adrenalectomized rats given salt or desoxycorticosterone acetate is reaffirmed. The dicarboxylic amino acids are decreased and alanine is increased in the livers of adrenalectomized rats given Compound E. The changes in amino acid concentration and distribution in muscle were not impressive. The relation of these findings to current theories is discussed.

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THE REACTION OF MALE FROGS TO EPINEPHRINE[1]

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WITH the exception of a few widely scattered reports (Galli-Mainini, 1947a, 1947b, 1947c; Houssay, 1947), almost all the information available today on the hormonal reactivity of male frogs and toads pertains to the effect of gonadotropins on these amphibia (Robbins, Parker and Bianco, 1947; Galli-Mainini, 1947a, 1948; Wiltberger and Miller, 1948; Miller and Wiltberger, 1948; Robbins and Parker, (literature) 1948). It has been already shown that a number of species of these male amphibia are available for endocrine assay work. The two male species most widely used at present for pregnancy diagnosis work are the South American toad, Bufo arenarum, and the North American spotted frog, the Rana pipiens. In pregnancy diagnosis, reports to date indicate that both animals appear to be quite satisfactory, giving virtually no false positives and very few false negatives (Galli-Mainini, 1948; Robbins and Parker, 1948). In contrast to the above two species the male Xenopus laevis, a South African frog, while a sensitive assay animal for gonadotropin of pituitary origin, has proved to be of little value in pregnancy diagnosis. While it reacts to gonadotropin of chorionic origin and thus faithfully detects pregnancy, it at the same time, however, gives many false positive reactions in women known to be not pregnant (unpublished work). Repeated efforts to isolate the substance or substances in urine producing these false positive reactions have been completely unsuccessful. No correlations have been drawn between these false positives and the diet of the patient, the ingestion of drugs or the phase of the menstrual cycle. It is of interest that these reactions were encountered only in girls having regular normal menses, not in patients with amenorrhea. A nonspecific alarm reaction to noxious agents contained in the urine with the release of adrenal hormones was considered as the possible basis of these false positive reactions. Accordingly, adrenocorticotropic hormone, desoxycorticosterone acetate, dehydro-isoandrosterone and epinephrine were administered subcutaneously to male Xenopus laevis and a

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rapid, strongly positive reaction to the epinephrine ensued. The corticotropic hormone, as well as the steroids, evoked no response. This reaction to epinephrine, while of great interest, seemed unlikely to be the rational explanation of the false positive tests encountered in the Xenopus laevis, since, to the best of our knowledge, epinephrine has never been identified in urine. However, the speed of the reaction and the simplicity of demonstrating this response suggested the possible applicability of this technic for the bio-assay of epinephrine preparations. Studies, therefore, on the reactivity of the male Rana pipiens and Xenopus laevis to epinephrine, as well as to various other autonomic drugs, form the basis of this present report.

EXPERIMENTAL DATA

Throughout these assay experiments adult male frogs procured from commercial collectors were used. The specimens of Xenopus laevis, had been gathered from their native habitat, South Africa, and weighed between 35 and 55 grams. The Rana pipiens species of frog were of domestic origin, weighing approximately 35 to 45 grams, and were for the most part kept in artificial hibernation throughout the year after being collected during the summer months.

These studies carried on throughout a year found the Xenopus laevis satisfactory for adrenalin assay at all times; the Rana pipiens, on the contrary, appeared to pass through a phase of adrenalin nonreactivity during the months of June, July and August. The basis of this transient refractoriness is totally obscure. All the preparations were used in aqueous form and were administered subcutaneously to the amphibia into the dorsal lymph sac. Reactions to these drugs, the emission of sperm from the cloacal tract, were followed by microscopic examination of the cloacal fluid, using the technic previously described (Robbins, Parker and Bianco, 1947).

Epinephrine of natural origin (Parke Davis, 1–1000 and Lederle, 1–1000) was administered to male amphibia, Rana pipiens, Xenopus laevis and Bufo arenarum, in doses of 0.1 cc. (0.1 mg.). The first two species reacted within one hour, the Bufo arenarum remaining negative indefinitely. No other effects of this drug on the animals were noted. To exclude the possibility that this positive reaction was caused by some side effect of contained animal substances present in the naturally derived epinephrine, synthetic hormone (Burroughs-Wellcome, 1–100) was likewise employed. Doses of 0.1 mg. of this synthetic preparation were equally effective in producing a positive response in the Rana pipiens and Xenopus laevis.

By the use of serial dilution technics it could be shown that both species of frogs, Xenopus laevis and Rana pipiens, would react to extremely small doses of epinephrine varying with individual animals between 0.01 mg. and 0.001 mg. Moreover, the reaction to this amount of drug remained remarkably constant for large numbers of adult males of both species, despite variations in the body weight of individual animals.

In an effort to determine whether this reaction to epinephrine was essentially the effect of the Sympathin "E" or "I" content, commercial preparations of these fractions were given to both types of frogs. The source of Sympathin I was Isuprel, 1–200 (Winthrop-Stearns); Arterenol, 0.1 mg., was used as a source of the "E" fraction. Both substances produced positive results in the doses indicated in the table which follows. A wide variety of other adrenergic drugs was administered to these amphibia with the results indicated in Table I.

TABLE 1

Substance	Xenopus laevis		Rana pipiens	
	Dose	Reaction[1]	Dose	Reaction[1]
Epinephrine (Parke Davis, 1–1000)	0.01 mgm.	++++	0.01 mgm.	++++
Epinephrine (Lederle, 1–1000)	0.001 mgm.	++++	0.003 mgm.	++++
Epinephrine (synthetic Burroughs-Wellcome, 1–100)	0.001 mgm.	++++	0.001 mgm.	++++
Isuprel (Sympathin I)	0.1 mgm.	++++	0.1 mgm.	++++
L-Arterenol (Sympathin E)			0.4 mgm.	++++
Neosynephrine	5.0 mgm.	0	25.0 mgm.	+++
Benzedrine sulfate	1.0 mgm.	++++	1.0 mgm.	0
Ephedrine sulfate	5.0 mgm.	++	5.0 mgm.	0
Mecholyl chloride	5.0 mgm.	0	5.0 mgm.	0
Eserine sulfate	0.64 mgm.	0	0.64 mgm.	0
Pilocarpine	4.0 mgm.	0	4.0 mgm.	0
Shock reaction to trauma		0		

[1] The reactions were graded 1+ to 4+ as follows:
+ =single sperm per high power field.
++ =several sperm per high power field.
+++ =most high power fields containing sperm too numerous to count.
++++ =all high power fields containing sperm too numerous to count.

Painful stimuli as well as crush injury to the hind extremity produced no emission of sperm, and in addition the administration of Carcholin (Merck), 0.1 mg., presumably stimulating the release of endogenous adrenalin in eserinized animals, evoked no response. Larger doses of Carcholin proved toxic to these animals.

In an effort to determine whether epinephrine was producing its effect through the pituitary, hypophysectomy by the method of Hogben (1923) was performed on several animals. The hypophysectomized amphibia reacted to epinephrine equally as well as the normal controls.

DISCUSSION

From observations on several hundreds of male Xenopus laevis and Rana pipiens it is apparent that these amphibia react to subcutaneously administered epinephrine by the emission of sperm. This response occurs rapidly, almost invariably within one hour after the administration of the drug. It is a readily demonstrable reaction as well as a remarkably constant one. The fact that graded doses down to a level of 0.01 to 0.001 of a mg. of epinephrine produce a response, doses below this level being inactive, strongly suggests some definite threshold phramacologic action of the drug. The positive reaction of Xenopus laevis to epinephrine carries over to such closely allied drugs as benzedrine and ephedrine, suggesting the possibility that some common chemical fraction or radical is the actual effective agent. It is interesting to note that some species difference exists between the Xenopus laevis and Rana pipiens in their reactivity to other ad-

renergic drugs since the latter type, Rana pipiens, fails to respond to any drugs other than epinephrine and neosynephrine. The reason for these differences is totally obscure. Both Sympathin I and Sympathin E produce positive reactions in the Rana pipiens. With the Xenopus laevis a positive reaction was obtained with Sympathin I, insufficient "E" being available for testing on this frog. None of the cholinergic drugs (acetyl Beta choline, acetyl choline or eserine) proved to be effective in producing a reaction, an observation in keeping with the apparent specificity of this response to adrenergic drugs.

The mechanism by which adrenalin and its related compounds produce emission of sperm in these frogs is extremely intriguing. At the present time, it has not yet been elucidated and work on this problem continues. Several aspects, however, seem worthy of note. The activity of so-called Sympathin I and E strongly suggests that epinephrine may be producing its effects by upsetting some finely balanced mechanism, either by the release of some block, such as some sphincteric cut-off, or by exciting some ejaculatory mechanism which overpowers the blocking mechanism. It is clear that the response of these male frogs to adrenalin does not represent simply a nonspecific reaction to bodily insult since noxious stimuli, pain and crushing injuries fail to produce the emission of sperm. The absence of a reaction to Carcholin, implying that endogenous adrenalin is ineffective in evoking the same response as exogenous adrenalin, may simply indicate failure of the effective release of the epinephrine by this drug. On the other hand, it is entirely possible that insufficient adrenal tissue is present in the frog to produce effective amounts of epinephrine. Microscopic search of the kidneys of these amphibia revealed only apparent adrenal cortical cells, never any tissue suggestive of adrenal medulla. In support of this possibility is the existence of a not inconsiderable controversy over the actual presence of a functioning adrenal gland in many amphibia. Markee et al. (1948) have shown that in the rabbit direct intrapituitary installation of adrenalin will produce the release of luteinizing gonadotropin. To rule out the possibility that these adrenergics might be thus acting on the genital system through the pituitary, hypophysectomy was performed on numerous frogs without effect on the reaction to epinephrine or benzedrine. Removal of the pituitary failed to alter the sensitivity of these amphibia to these drugs. In conclusion, therefore, at the present writing the mode of action of these epinephrine substances remains obscure.

Despite the lack of knowledge as to the "modus operandi" the empiric observation that male Rana pipiens and Xenopus laevis will respond to a relatively constant dose of epinephrine provides a most valuable tool for pharmacologic assay, providing a substitute for present laborious blood-pressure studies on dogs. Injection of a series of graded dilutions of epinephrine of unknown strength into male frogs will rapidly disclose the approximate content of the drugs in

these unknown solutions, since it can be reasonably certainly assumed from this study that the endpoint dose contains 0.01–0.001 mg. of epinephrine. Thus unknown solutions may be titered by the use of male frogs to reasonably close tolerances within 1–2 hours. The simplicity of performance of this technic, as well as its economy and ease of maintaining colonies of frogs, renders this procedure of real value.

Several limitations deserve re-emphasis. In summer months the sensitivity of Rana pipiens, derived from native habitats, to adrenalin is considerably lowered, rendering this use of these frogs during this time undesirable, a defect not encountered in the Xenopus laevis. Whether Rana pipiens kept in enforced hibernation from the previous summer will show this seasonal insensitivity to adrenalin is not known. The second point of caution is that the two species of toads, namely, Bufo arenarum Hensel and Bufo fowleri, both fail to respond to adrenalin. Galli-Mianini (1948) in a recent report confirms this observation in the Bufo arenarum. This species difference in reactivity to adrenalin is in itself a most interesting point, suggesting possible differences in the makeup of the endocrine or genital systems between various species of amphibia.

SUMMARY

It has been shown that certain male amphibia respond to the subcutaneous administration of epinephrine by the emission of spermatozoa, the South African Xenopus laevis being reactive throughout the year, the Rana pipiens passing through a refractory period during the summer months of June, July and August. This reaction to epinephrine appears to be a remarkably constant one in which most male Xenopus laevis and Rana pipiens adults, despite some variation in body weight, always respond to dosage levels between 0.01 and 0.001 mgms. Synthetic as well as naturally derived epinephrine are equally effective and hypophysectomy in no way alters the reactivity of the animal. With the Xenopus laevis this epinephrine reactivity is carried over to the adrenergic drugs, benzedrine and ephedrine.

The possible practical application of this male amphibian reaction to the bio-assay of epinephrine preparations is discussed.

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ASSOCIATION NOTICE

ANNOUNCEMENT OF THE 1949 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-First Annual Meeting of The Association for the Study of Internal Secretions will be held in the Chalfonte-Haddon Hall, Friday and Saturday, June 3, and 4, 1949, in Atlantic City, New Jersey.

We are informed by the hotel management that reservations will be difficult to secure on short notice; therefore, members are urged to make reservations at once with Chalfonte-Haddon Hall, giving time of arrival and length of stay in Atlantic City.

The scientific sessions will be held in the Viking Room, as formerly, and registration will be on the same floor. The annual dinner will be held in the Rutland Room, Friday, June 3rd. at 7 P.M., preceded by cocktails in the same room.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Doctor J. S. L. Browne, Royal Victoria Hospital, Montreal 2, Canada, not later than March 1, 1949. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program.

Nominations for the Squibb and Ciba Awards and the Ayerst, McKenna and Harrison Fellowship should be made on special application forms, which may be obtained from the Secretary-Treasurer, Doctor Henry H. Turner, 1200 North Walker, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1949.

POSTGRADUATE COURSE IN ENDOCRINOLOGY

A postgraduate course in Endocrinology, sponsored by the Association for the Study of Internal Secretions, will be held at the Skirvin Hotel in Oklahoma City, February 21–26, 1949.

The faculty will consist of outstanding clinical and research endocrinologists of the United States and Canada. The program will consist of clinics and demonstrations and will be a practical one of equal interest to those in general medicine and the specialists.

The fee will be $100 for the entire course and applications will be accepted in the order received. Applications should be directed to Henry H. Turner, M. D., Secretary-Treasurer, 1200 North Walker, Oklahoma City, Oklahoma.

ASSOCIATION AWARDS FOR 1949

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for $1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00.

THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed $2,500.00 The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence or scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.